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Differences in the Control of Virus mRNA Splicing during Permissive or Abortive Infection with Influenza A (Fowl Plague) Virus

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SUMMARY

Spliced transcripts of influenza A (fowl plague) virus (FPV) RNA (vRNA) segments 7 and 8 accumulate to a much greater extent during non-productive infection of mouse L cells, than they do during productive infection in primary chick embryo fibroblasts (CEF). Virus-specific protein synthesis, or a consequent event in virus replication appears necessary to promote splicing of vRNA segment 8-encoded mRNAs in both cell types, and of vRNA segment 7-encoded mRNAs in CEF. In L cells, however, splicing of the segment 7-encoded mRNAs seems to be independent of such virus-specific control. This observation is discussed in relation to the defect in expression of vRNA 7 which has been observed previously in FPV-infected L cells, and which is thought to account for the failure of virus replication.

INTRODUCTION

Each of the two smallest virion RNA (vRNA) segments (7 and 8) of influenza A virus contains two overlapping genes (Porter *et al.*, 1980; Lamb & Lai, 1980, 1981; Winter & Fields, 1980; Winter *et al.*, 1981; Allen *et al.*, 1980; Baez *et al.*, 1980, 1981; McCauley *et al.*, 1982) which are expressed during virus replication from different species of mRNA. In the infected cell, vRNAs 7 and 8 are transcribed initially into mRNAs (7 and 8) which represent virtually full-length copies of their templates (Hay *et al.*, 1977*a*) and which encode the 28000 mol. wt. (28K) structural matrix protein (M) and the 26K non-structural protein (NS_1) respectively (Ritchey *et al.*, 1976; Scholtissek *et al.*, 1976; Inglis *et al.*, 1977).

In addition, infected cells contain one smaller vRNA 8-specific polyadenylated RNA (Inglis *et al.*, 1979) and two smaller vRNA 7-specific polyadenylated RNAs (Inglis & Brown, 1981; Lamb *et al.*, 1981) which are assumed to arise through splicing of the full-length transcripts (see Fig. 1), since they are complementary to non-contiguous regions on their respective vRNA templates (Lamb & Lai, 1980; Inglis & Brown, 1981; Lamb *et al.*, 1981). The small vRNA 8-encoded mRNA (mRNA 9), which encodes the NS_2 protein (11K to 14K) (Inglis *et al.*, 1979), contains sequences identical to the 5′-terminal 56 nucleotides and the 3′-terminal 361 nucleotides of the NS_1 mRNA (Lamb & Lai, 1980) while the two small segment 7-encoded RNAs (mRNAs 10 and 11) comprise the 3′-terminal 287 nucleotides of the matrix mRNA spliced to either 12 (for mRNA 11) or 51 (for mRNA 10) nucleotides from the 5′ end (Lamb *et al.*, 1981). The larger of these two species encodes the recently identified M_2 protein (Lamb & Choppin, 1981), but the coding function of the smaller mRNA remains unknown.

The enzyme activity responsible for processing of the virus mRNAs has not yet been identified, but since the sequences surrounding the splice points in both the NS_1 and M mRNA resemble those which signal splicing in normal cellular mRNAs (Lamb & Lai, 1980; Lamb *et al.*, 1981), it seems likely that the enzyme is of cellular origin. In support of this notion, at least some of these signals were utilized correctly during infection with a recombinant simian virus (SV) 40 virus which had been manipulated to contain a DNA copy of influenza vRNA segment 7 (Lamb & Lai, 1982). It seemed possible therefore that certain host cell-specific differences in influenza virus gene expression which had been documented previously (Bosch *et al.*, 1978; Valcavi *et al.*,

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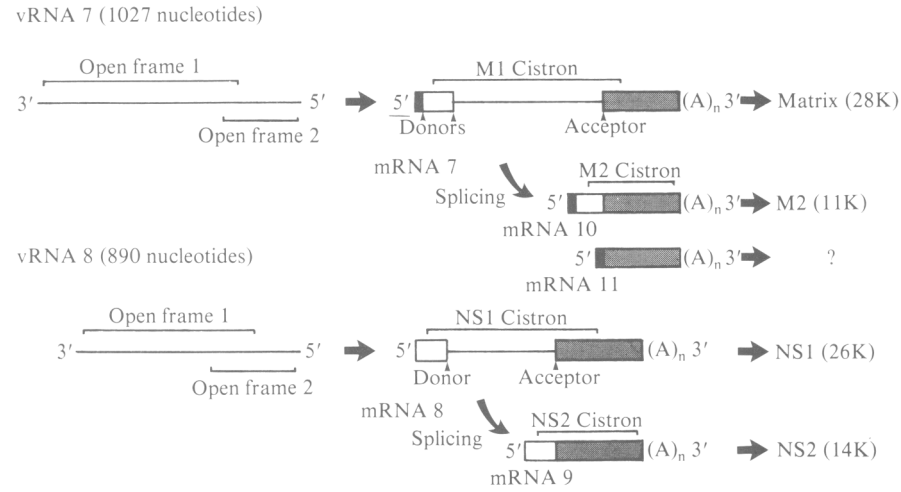

Fig. 1. Scheme for expression of spliced and unspliced mRNAs from vRNA 7 and vRNA 8 of influenza virus (Lamb & Lai, 1980; Lamb *et al.*, 1981; Lamb & Choppin, 1981; Inglis *et al.*, 1979; Inglis & Brown, 1981).

1978; Lamb & Choppin, 1978) might be explained by variation in the efficiency of virus mRNA splicing. We report here that for the avian influenza A virus, fowl plague (FPV), the proportion of spliced mRNAs which accumulate in infected L cells is much greater than in primary chick embryo fibroblasts (CEF) and that control of this splicing process occurs differently in the two cell types. In CEF, the synthesis of new virus proteins appears necessary either directly or indirectly to promote splicing of both vRNA 7- and vRNA 8-encoded mRNAs. In L cells, efficient splicing of vRNA 7-encoded mRNA does not exhibit this dependence. These observations may help to explain defects in the expression of virus proteins which have been implicated in the failure of FPV to replicate in certain cell types.

METHODS

Cells and virus. Influenza A/FPV/Rostock strain was propagated in fertile hens' eggs, assayed, and used to infect tissue culture cells as described previously (Inglis *et al.*, 1976). Primary CEF cultures were prepared as described by Borland & Mahy (1968) and L929 cells were obtained, as a continuous line, from Flow Laboratories.

Analysis of infected cell protein synthesis. Cells were pulse-labelled with [^{35}S]methionine (20 μCi/ml) in methionine-free medium before harvesting, and were analysed on 17·5% SDS–polyacrylamide gels as described previously (Inglis *et al.*, 1976).

Preparation of FPV-infected cell RNA. Infected cells were fractionated into nuclei and cytoplasm, and cytoplasmic RNA was extracted as described previously (Inglis *et al.*, 1977). Nuclei were lysed in high salt buffer (0·5 M-NaCl, 0·05 M-$MgCl_2$, 0·01 M-Tris–HCl pH 7·4), and incubated with bovine pancreatic deoxyribonuclease I (50 μg/ml) for 5 min at 37 °C before RNA extraction as before. Poly(A)-containing RNA was purified by oligo-(dT)–cellulose chromatography (Inglis & Mahy, 1979). Where ^{32}P-labelled RNA was required, cells were pre-starved of phosphate for 16 h then labelled with [^{32}P]orthophosphate (1 mCi/ml) for 3 h before, as well as during, infection. Labelling of cells with [^{3}H]uridine was carried out in serum-free medium at a concentration of 200 μCi/ml. Poly(A) was removed from mRNA using ribonuclease H in the presence of oligo(dT) as previously described (Inglis *et al.*, 1980).

Synthesis of complementary DNA. DNA copies of infected cell mRNAs were synthesized using reverse transcriptase, oligo$(dT)_{12-18}$ as primer, and [α-^{32}P]dTTP as an internal label. Reaction conditions and procedures for processing of samples for gel electrophoresis were as detailed elsewhere (Inglis *et al.*, 1980).

Selection of virus-specific mRNAs and cDNAs using immobilized plasmid DNA. Bacterial plasmids containing sequences from vRNA segments 7 and 8 of FPV were immobilized on nitrocellulose filters and hybridized with labelled cDNAs or mRNAs as before (Inglis & Brown, 1981). The plasmids used were all based on pBR322 (Bolivar *et al.*, 1977) and their virus-specific information content was as follows: pFPV 71 (Caton & Robertson, 1980), a complete copy of vRNA 7; pFPV 71B (Inglis & Brown, 1981), the 3′-terminal 293 nucleotides from

vRNA 7; pISI 86 (Roditi, 1983), a complete copy of vRNA 8; pFPV 82A (prepared in this laboratory by D. Smith), the 3′-terminal 51 nucleotides of vRNA 8.

Nucleic acids that hybridized specifically to immobilized DNA were eluted, recovered, and analysed on Tris-borate–EDTA-buffered 7·5% polyacrylamide gels as described by Inglis & Brown (1981).

RESULTS

Expression of vRNA segment 7- and segment 8-encoded polypeptides in CEF and L cells

Primary CEF, which are permissive for FPV replication (Breitenfeld & Schafer, 1957), and L cells which do not support FPV growth (Franklin & Breitenfeld, 1959) were pulse-labelled with [^{35}S]methionine at various times after infection, and analysed by gel electrophoresis (Fig. 2*a*, *b*). Virus-specified protein synthesis in CEF (Fig. 2*a*) conforms to a well-documented pattern in which synthesis of NP and NS_1 is amplified early in infection, while M and haemagglutinin (HA) protein syntheses are increased selectively at later times (Skehel, 1973; Inglis *et al.*, 1976; Bosch *et al.*, 1978; Inglis & Mahy, 1979). The smaller of the two vRNA 8-encoded proteins, NS_2, appears later than NS_1, and its synthesis remains relatively low throughout infection.

The pattern of virus protein synthesis in the L cells differed from that in CEF in two main respects. Firstly, the degree of amplification of M protein synthesis was much less marked, as Bosch *et al.* (1978) have noted before, and secondly, synthesis of NS_2, although initially less than that of NS_1, eventually exceeded that of all the other virus proteins. In these experiments we were unable consistently to detect a protein with characteristics of the second vRNA 7 gene product, M_2 (Lamb *et al.*, 1981) despite using [^{35}S]cysteine as a radioactive precursor, and so the characteristics of its synthesis and its relative abundance remained uncertain.

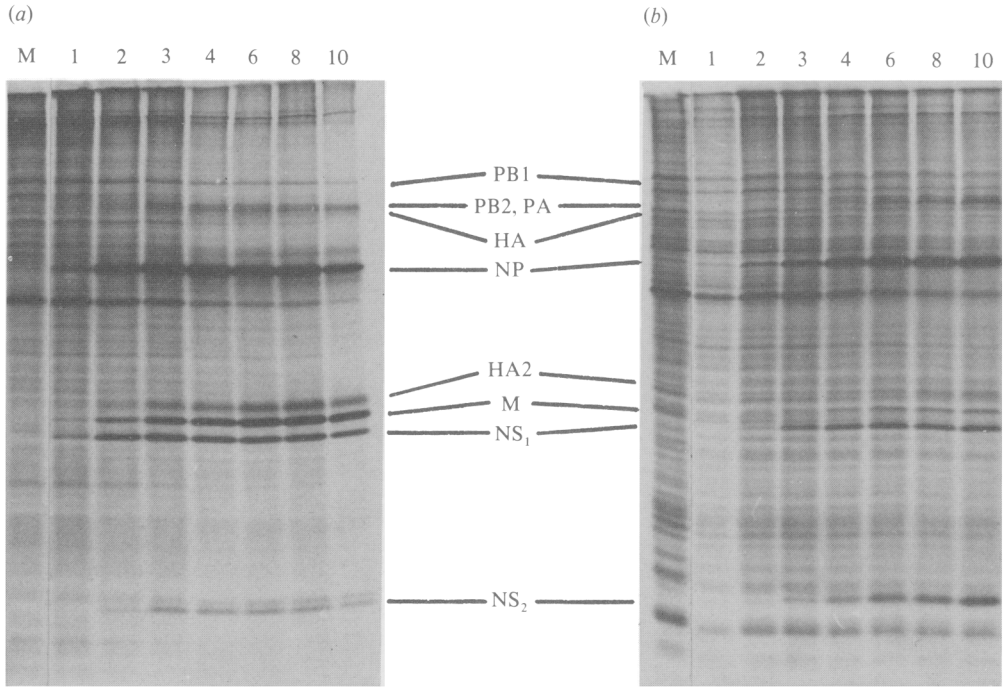

Fig. 2. Time course of polypeptide synthesis in FPV-infected CEF (*a*) and L cells (*b*). Infected and mock-infected cells were labelled with [^{35}S]methionine for 30 min immediately before harvesting at the times (h post-infection) indicated above each lane; M, mock-infected. Approximately equal amounts of cell protein were loaded in each lane, separated on a 17·5% polyacrylamide gel and detected by autoradiography.

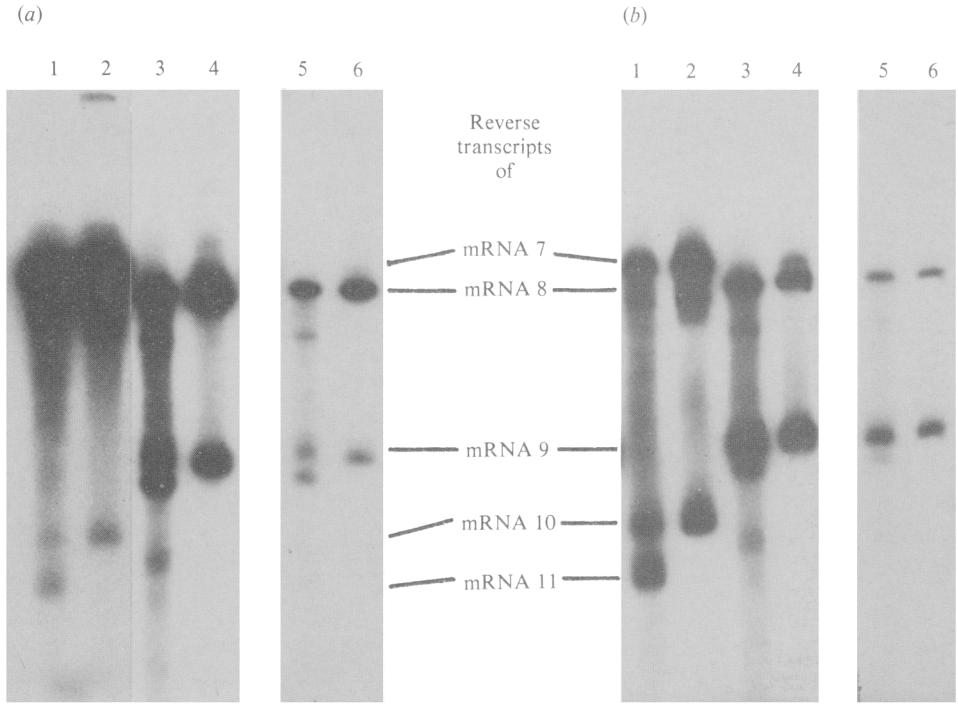

Fig. 3. Hybrid selection of vRNA 7-specific (lanes 1 and 2) and vRNA 8-specific (lanes 3 to 6) reverse transcripts of mRNA from FPV-infected CEF (*a*) and L cells (*b*). Cytoplasmic poly(A)-containing RNA was extracted 5 h post-infection, reverse-transcribed using [α-^{32}P]dTTP as a radioactive tracer, and the reaction products were annealed with plasmid DNA which had been immobilized on nitrocellulose discs (Methods). The plasmids used were the vRNA 7-specific pFPV 71 (lane 1) and pFPV 71B (lane 2), and the vRNA 8-specific pISI 86 (lane 3) and pFPV 82A (lane 4). Sequences that hybridized specifically were eluted, separated on a 7·5% polyacrylamide gel and detected by autoradiography. Lanes 5 and 6 represent shorter exposures of lanes 3 and 4 respectively.

Analysis of vRNA segment 7- and segment 8-encoded mRNAs in CEF and L cells

Increased synthesis of NS_2 and decreased synthesis of M protein in L cells might be explained by an increase in the proportion of spliced to unspliced mRNAs which accumulate in the cytoplasm, and so we next investigated the relative amounts of the mRNAs specified by vRNAs 7 and 8 in the two cell types. This was accomplished using a filter-hybridization technique described previously (Inglis *et al.*, 1980; Inglis & Brown, 1981) in which cloned virus-specific DNA was used to select, for gel analysis, complementary DNA copies of specific virus mRNAs. Radioactively labelled reverse transcripts of mRNA from infected CEF and L cells were hybridized with plasmids containing either full-length copies of vRNAs 7 and 8 (pFPV 71 and pISI 86 respectively) or plasmids which included only the 3′-terminal sequences of these segments (pFPV 71B and pFPV 82A respectively). The latter plasmids were used since they select cDNA copies of the corresponding spliced mRNAs yet exclude all but the largest incomplete transcripts of the full-length mRNAs (e.g. Fig. 3*a*, lane 5 contains two prominent incomplete cDNA copies of mRNA 8, that are not present in lane 6). The exception to this is mRNA 11 which has insufficient homology with the 3′ end of vRNA 7 to permit efficient hybridization, and which therefore is not selected by pFPV 71B.

Fig. 3(*a*, *b*) shows that in the cytoplasm of the L cells, the proportion of both vRNA 7- and vRNA 8-specified mRNAs that are present in a spliced form is much higher than in CEF. There was no preferential accumulation of the unspliced full-length mRNAs in the nuclei of these cells (data not shown), suggesting that the altered proportions of the segment 7- and 8-specific mRNAs, which presumably account for the altered pattern of protein synthesis observed in Fig. 2, were due to an increased level of splicing.

Temporal control of virus mRNA splicing in L and CEF cells

Next we monitored, by the same method as before, the accumulation throughout infection of the spliced virus mRNAs in L and CEF cells (Fig. 4). Analysis of vRNA 8-specific mRNAs (Fig. 4*b*) indicated that in both cell types, mRNA 8 (NS_1 mRNA) was the major species present early in infection, but that the relative proportion of mRNA 9 increased later (compare for example the lanes marked 2 and 6 for both CEF and L cells), particularly in L cells where mRNA 9 became eventually more abundant than mRNA 8.

The pattern of accumulation of vRNA 7-specific mRNAs in CEF (Fig. 4*a*, left-hand panel) was similar in that the spliced mRNAs (10 and 11) appeared in relatively small amounts early in infection, and there was a slight increase in the proportion of spliced to unspliced mRNAs at later times. However, the ratio of the two spliced mRNAs changed throughout infection, mRNA 10 predominating at early times while mRNA 11 became more abundant later. In contrast, in L cells mRNAs 10 and 11 appeared as early as mRNA 7, and there was little change in their proportion relative to each other throughout infection.

It was of interest to know whether the changes in the relative amounts of mRNAs 10 and 11 present during infection of CEF were the result of altered rates of synthesis or perhaps different rates of turnover of the two species. These possibilities were examined by pulse-labelling infected CEF with [^{3}H]uridine at different times after infection. After a 2 h labelling period, total cytoplasmic RNA was extracted and analysed for the presence of labelled mRNAs 10 and 11 directly by hybrid selection and gel electrophoresis following removal of poly(A) (Fig. 5).

It was not necessary to fractionate the labelled RNA by oligo(dT)–cellulose chromatography for this experiment, since infected cells do not contain unpolyadenylated equivalents of mRNAs 10 and 11 (unpublished observations). During the first 2 h of infection (lane 1), the two spliced mRNAs were apparently synthesized in similar amounts, but thereafter there was a relative decrease in production of mRNA 10, so that by 4 to 6 h post-infection (lane 3) mRNA 11 was the only detectable species. Analysis of nuclear RNA from these cells by the same procedure (lanes 5 and 6) indicated that there was no preferential accumulation of mRNA 10 in the nucleus later in infection, suggesting that the splice donor site nearest the 5′ end of mRNA 7 (see Fig. 1) was favoured later in infection.

Virus mRNA splicing in L cells and CEF in the absence of protein synthesis

It seemed possible that the changes in the overall amounts and in the pattern of splicing which occurred during infection might depend on the synthesis of new virus proteins. The production of spliced mRNAs was therefore investigated in L cells and CEF which had been infected in the presence of the protein synthesis inhibitors cycloheximide and anisomycin (Fig. 6). Under these conditions, virus-specific RNA synthesis is limited to transcription of the infecting genome RNA into mRNA by the endogenous virion transcriptase (Hay *et al.*, 1977*b*). L cells and CEF cultures were incubated with both protein synthesis inhibitors, in the presence of [^{32}P]orthophosphate, from 1 h before infection until 5 h after infection, when cytoplasmic RNA was extracted. The RNA was treated with ribonuclease H to remove poly(A) tails and was analysed directly, as before, for the presence of segment 7- and 8-specific mRNAs (lanes 3 to 6).

In CEF infected in the presence of the drugs (lanes 3 and 4) the relative production of spliced mRNAs, particularly of mRNAs 10 and 11, was lower than in cells which had not been treated with the inhibitors (lanes 1 and 2, and Table 1). In further experiments where the amounts of vRNA 7- and vRNA 8-encoded mRNAs were measured through reverse transcription and hybrid selection, the drugs exerted an equivalent, or more pronounced inhibitory effect on the appearance of the spliced mRNAs. This reduction was not apparently a direct effect of the inhibitors on splicing in CEF, since it was not observed when the drugs were added after infection had been established but prior to radioactive labelling (lanes 7 and 8, and Table 1). It seems therefore that protein synthesis, presumably virus-specified, is necessary to promote splicing of virus mRNAs in the infected CEF cell. In L cells, splicing of segment 8 transcripts was similarly reduced by drug treatment (e.g. compare Fig. 6, lane 6 with Fig. 3*b*, lane 5) but in contrast, production of mRNAs 10 and 11 appeared unaffected (e.g. compare Fig. 6, lane 5 with

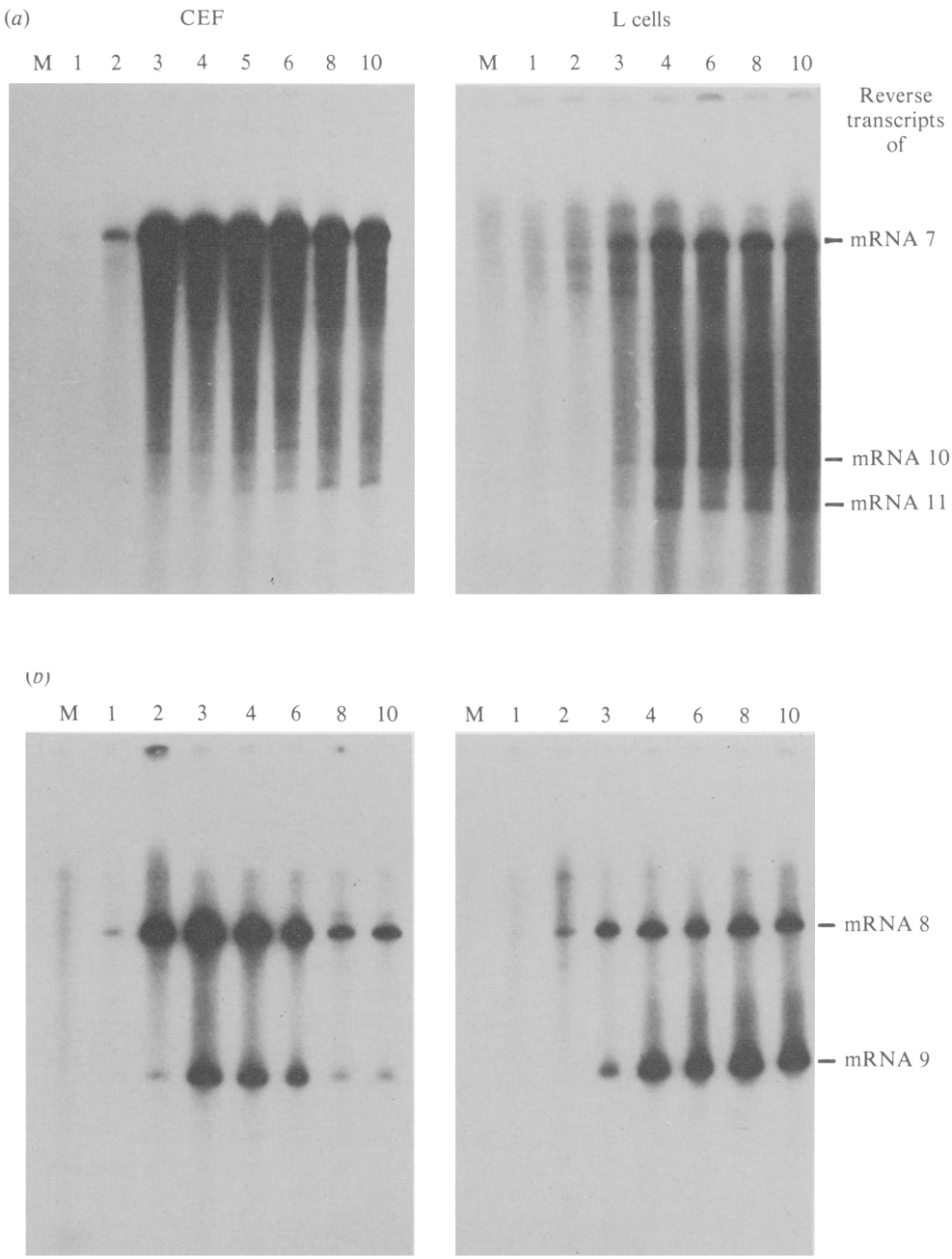

Fig. 4. Time course of accumulation of (*a*) vRNA 7-specific and (*b*) vRNA 8-specific mRNAs in FPV-infected CEF (left-hand panels) and L cells (right-hand panels), monitored by cDNA synthesis. Poly-(A)-containing RNA was extracted from the cytoplasm of infected cells and equal amounts were reverse-transcribed in the presence of [^{32}P]dTTP. The products were annealed with nitrocellulose filters carrying either vRNA 7-specific plasmid DNA, pFPV 71 (*a*) or vRNA 8-specific plasmid DNA, pFPV 82A (*b*). Sequences that hybridized specifically were eluted, separated on a 7·5% polyacrylamide gel, and detected by autoradiography. The numbers above each lane refer to the times (h post-infection) at which RNA was prepared; M, mock-infected.

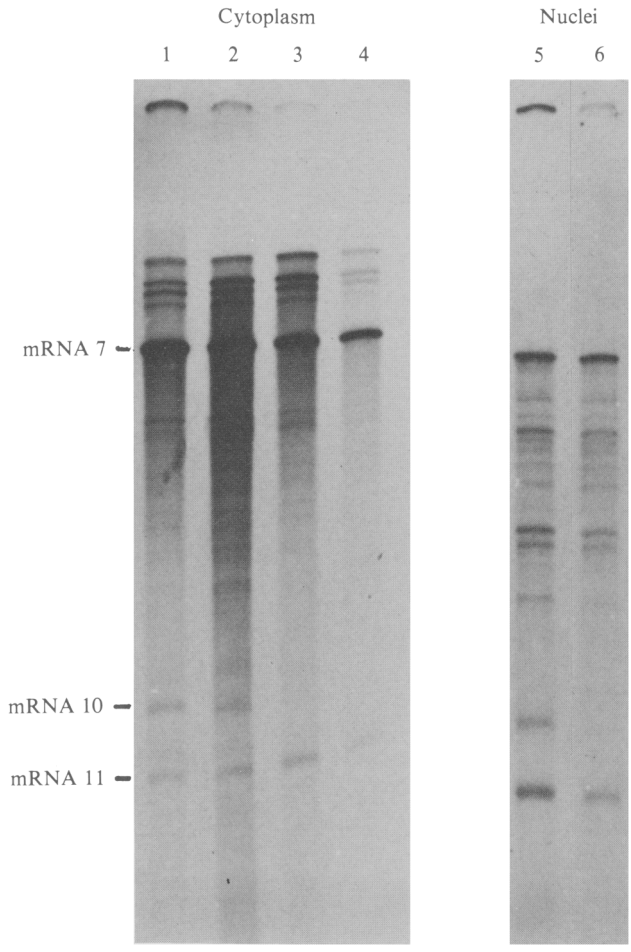

Fig. 5. Synthesis of mRNAs 10 and 11 in FPV-infected CEF. Infected cells were incubated with [^{3}H]-uridine (Methods) from 0 to 2 h (lane 1), 2 to 4 h (lanes 2 and 5), 4 to 6 h (lane 3) and 6 to 8 h (lanes 4 and 6) after infection and fractionated into nuclei and cytoplasm immediately after labelling. Total RNA was extracted from the cytoplasm (lanes 1 to 4) and where indicated the nuclei of these cells (lanes 5 and 6), and hybridized with nitrocellulose discs carrying vRNA 7-specific plasmid DNA (pFPV 71). Hybridized RNAs were eluted, treated with ribonuclease H in the presence of oligo(dT) in order to remove poly(A), separated on a 7·5% polyacrylamide gel, and detected by fluorography.

Fig. 3*b*, lane 1), suggesting that in these cells virus-specified protein synthesis was not required to allow efficient splicing of segment 7 transcripts.

DISCUSSION

Our results suggest that in FPV-infected CEF, splicing of vRNA 7-and 8-encoded mRNAs is dependent on virus-specific factors since the presence of protein synthesis inhibitors from the start of infection inhibited the accumulation of spliced mRNAs. This was not a direct effect of the inhibitors on the efficiency of splicing, because administration of the drugs after infection had commenced no longer exerted the same effect, and so it would appear that virus-specific proteins, or more probably events in the replication cycle which are dependent on the synthesis of new virus proteins, are necessary to promote splicing of virus-specific mRNAs in the infected cell. This implication is supported by previous observations that RNA from CEF which had

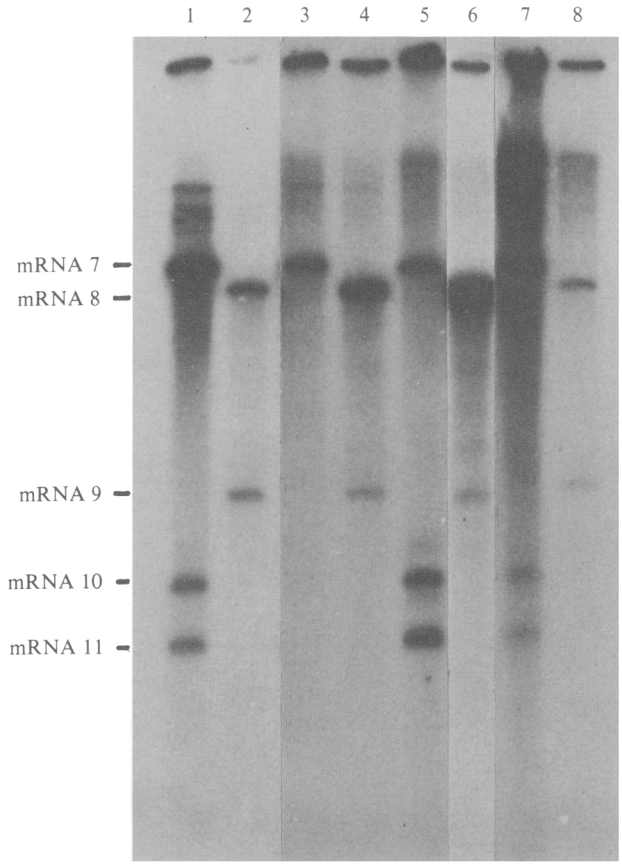

Fig. 6. Synthesis of spliced mRNAs in FPV-infected CEF (lanes 3 and 4) and L cells (lanes 5 and 6) in the absence of virus protein synthesis. Cells were incubated with [^{32}P]orthophosphate from 6 h before infection, and cycloheximide (100 μg/ml) plus anisomycin (20 μg/ml) from 1 h before infection until the time of harvesting at 5 h post-infection. Total cytoplasmic RNA was extracted immediately and hybridized with nitrocellulose filters carrying vRNA 7-specific plasmid DNA (pFPV 71, lanes 3 and 5) or vRNA 8-specific plasmid DNA (pISI 86, lanes 4 and 6). Sequences that hybridized were eluted, treated with ribonuclease H in the presence of oligo(dT) to remove poly(A), separated on a 7·5% polyacrylamide gel and detected by autoradiography. Lanes 1 and 2: CEF were infected, labelled, and RNA was extracted at 5 h as before except that no drugs were added. Poly(A)-containing RNA was then prepared and hybridized with either vRNA 7-specific DNA (lane 1) or vRNA 8-specific DNA (lane 2) before ribonuclease H treatment and gel analysis as before. Lanes 7 and 8: infected CEF were incubated with cycloheximide plus anisomycin (concentration as before) from 2·5 h after infection, and with [^{32}P]orthophosphate from 5 h after infection, until 8 h when total cytoplasmic RNA was extracted as before. The RNA was hybridized with immobilized vRNA 7-specific DNA (lane 7) or vRNA 8-specific DNA (lane 8). Specifically bound sequences were eluted, ribonuclease H-treated, and analysed by gel electrophoresis as above.

been infected with influenza virus in the presence of cycloheximide failed to direct the synthesis of NS_2 in cell-free translation systems (Lamb *et al.*, 1978; Inglis, 1978). Also consistent with this notion, in our experiments the proportion of spliced transcripts in the cytoplasm of infected CEF, particularly those of vRNA 8 increased as infection progressed.

We do not know why splicing should be dependent on virus-specific factors in this system. From the work of others, it is clear that the splicing signals present on the virus mRNAs can be utilized by mammalian cell enzymes in the absence of other virus information. In cells infected with a recombinant SV40 virus in which part of the 'late' region had been replaced by a DNA copy of vRNA 7 (of a human strain of influenza virus), approximately half of the transcripts

Table 1. *Relative amounts of vRNA 7- and vRNA 8-encoded spliced and unspliced mRNAs produced in CEF in the presence and absence of protein synthesis inhibitors**

	Proportion of spliced : unspliced mRNA†		
	No inhibitors	Inhibitors present throughout infection	Inhibitors added after infection
mRNA 9 : mRNA 8	21·5 (lane 2)	4·8 (lane 4)	17·9 (lane 8)
mRNA 10 : mRNA 7	9·5 (lane 1)	<0·5‡ (lane 5)	–§
mRNA 11 : mRNA 7	5·7 (lane 1)	<0·5‡ (lane 5)	–§

* Lanes from the autoradiogram shown in Fig. 6 were scanned and the area under the relevant peaks computed, using a Beckman Du-8 spectrophotometer.

† The results are expressed as the percentage proportion of radioactivity in the spliced mRNA relative to that present in its unspliced equivalent. The gel lane from which the data were obtained is indicated in brackets after each value.

‡ The spliced mRNA was not detectable.

§ Accurate values could not be obtained owing to a high background of radioactivity (Fig. 6, lane 7).

from this region accumulated in a spliced form (Lamb & Lai, 1982), although this fraction did not include an equivalent of mRNA 10. In L cells and baby hamster kidney cells that carry stable integrated DNA copies of vRNA 8 from FPV, transcripts of these genes are spliced with still higher efficiency (M. Inglis, personal communication). It is possible that CEF cell splicing enzymes are unusual in their inability to process efficiently virus mRNAs without help from the virus proteins, but since the sequences surrounding splice sites in avian cell mRNAs do not appear to differ greatly from those found in mammalian cell mRNAs (Mount, 1982) this seems unlikely. A more plausible explanation is that in CEF, in the absence of virus protein synthesis, mRNA transcripts are synthesized or modified in such a way that the splicing machinery is avoided. Later in infection, when virus proteins are available the mode of synthesis or presentation of the transcripts is altered to allow a greater degree of processing.

It was evident in our experiments that the progress of infection influenced not only the overall amount of virus mRNA splicing, but also with regard to vRNA 7 transcripts, the specificity of the splice events. During the early phase, mRNA 10 and mRNA 11 were equally abundant, whereas later on, mRNA 11 was the predominant species. This shift appeared to be the result of a change in the relative rates of synthesis of the two mRNAs rather than a reflection of different turnover rates, suggesting that splice events involving the donor site nearest the 5′ end of mRNA 7 (see Fig. 1) are preferred later in infection. Similar changes in the pattern of virus mRNA splicing are thought to occur during infection with adenovirus (Chow *et al.*, 1979; Nevins & Wilson, 1981) but the mechanism by which they occur is unknown.

The spliced mRNAs encoded by vRNA segments 7 and 8 of FPV accumulated to a much greater extent, relative to their unspliced counterparts, in infected L cells as compared with infected CEF. Although we cannot entirely rule out the possibility that different rates of mRNA turnover are responsible for this effect, it seems most likely that it reflects an increased efficiency of splicing of these mRNAs in L cells. Such an increase might be the result of a higher affinity of L cell splicing enzymes for virus-specific substrate mRNAs, but an alternative possibility is that the substrates for splicing are more readily available in L cells than they are in CEF, as a result of differences in the rates or sites of their synthesis, or in their structural presentation. A surprising aspect of these experiments was that although L cells clearly contain relatively large amounts of mRNA 10, we were unable consistently to detect its protein product, M_2 (Lamb & Choppin, 1981) despite using [^{35}S]cysteine as a radioactive label. It may be that M_2 is unstable in these cells, or that translation of its mRNA is somehow prevented, but we cannot distinguish between these possibilities at present.

In FPV-infected L cells, the control over virus mRNA splicing exerted by virus-specific components appeared more complex than in CEF. Transcripts of vRNA 8 were inefficiently spliced under conditions of primary transcription. If infection was allowed to proceed normally,

the abundance of NS_2 mRNA, relative to NS_1 mRNA, increased markedly throughout infection, suggesting, as for infected CEF, a requirement for virus-specific protein synthesis to promote splicing. In contrast, vRNA 7 transcripts were processed as efficiently in the absence of virus protein synthesis as they were during a normal infection.

It is not clear why splicing of matrix mRNA appears to depend on virus-specific factors in CEF yet not in L cells, but this distinction could help to explain differences between the modes of virus replication in these two cell types. It is well established that L cells cannot support productive infection by FPV, yet allow a limited degree of virus gene expression (Franklin & Breitenfeld, 1959; Gandhi *et al.*, 1971). Bosch *et al.* (1978) showed that in L cells, the amplification in synthesis of the mRNA for M protein, which is a characteristic of the late stage in CEF infection (Hay *et al.*, 1977*b*; Inglis & Mahy, 1979), did not occur. The consequent reduction in synthesis of M protein was therefore advanced as a possible cause of inadequate virus assembly. More recently, Smith & Hay (1982) reported that the synthesis of vRNA 7 was also reduced in infected L cells and suggested therefore that the decreased production of M mRNA in these cells was due to a dearth of templates for its synthesis.

It is tempting to speculate that this defect in expression of vRNA 7 in L cells might be linked with the capacity of these cells to splice vRNA 7 transcripts in an apparently uncontrolled fashion. For example, accumulation of M protein might be required to promote synthesis of vRNA 7 late in infection, and so in infected L cells this positive regulation cycle might be interrupted. There is as yet no evidence to suggest that the influenza virus M protein has such a regulatory function, but the matrix protein of vesicular stomatitis virus has been implicated in control of virus-specific RNA synthesis (Clinton *et al.*, 1978; Carroll & Wagner, 1979).

From the results presented here and elsewhere, it is clear that the amplification of M protein synthesis in influenza virus-infected cells is dependent on host cell factors. Synthesis of M protein is specifically reduced in FPV-infected KB cells, as well as in L cells (Valcavi *et al.*, 1978) and occurs differently in different cell types infected with the same strain of human influenza virus (Lamb & Choppin, 1978). Furthermore, inhibitors of cell DNA function, such as actinomycin D and camptothecin, or pre-irradiation with u.v. light have a selective effect on the amplification of M protein synthesis in FPV-infected CEF (Minor & Dimmock, 1975, 1977; Mahy *et al.*, 1977; Inglis *et al.*, 1978). It is conceivable therefore that alterations in host cell-dependent splicing of vRNA 7 transcripts might provide a link between these phenomena.

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REFERENCES

ALLEN, H., McCAULEY, J., WATERFIELD, M. & GETHING, M. J. (1980). Influenza virus RNA segment 7 has the coding capacity for two polypeptides. *Virology* **107**, 548–551.

BAEZ, M., TAUSSIG, R., ZAZRA, J. J., YOUNG, J. F. & PALESE, P. (1980). Complete sequence of the influenza A/PR/8/34 NS gene and comparison with the NS gene of the A/Udorn/72 and A/FPV/Rostock/34 strains. *Nucleic Acids Research* **8**, 5845–5858.

BAEZ, M., ZAZRA, J. J., ELLIOTT, R. M., YOUNG, J. F. & PALESE, P. (1981). Nucleotide sequence of the influenza A/duck/Alberta/60/76 virus NS RNA: conservation of the NS_1/NS_2 overlapping gene structure in a divergent influenza virus RNA segment. *Virology* **113**, 397–402.

BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P., BETLACH, M. C., HEYNEKER, H. L., BOYER, H. W., CROSA, J. & FALKOW, S. (1977). Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.

BORLAND, R. & MAHY, B. (1968). Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity in cells infected with influenza virus. *Journal of Virology* **2**, 33–39.

BOSCH, F. X., HAY, A. J. & SKEHEL, J. J. (1978). RNA and protein synthesis in a permissive and an abortive influenza virus infection. In *Negative Strand Viruses and the Host Cell*, pp. 465–474. Edited by B. W. J. Mahy & R. D. Barry. London: Academic Press.

BREITENFELD, P. M. & SCHAFER, W. (1957). The formation of fowl plague virus antigens in infected cells as studied by fluorescent antibodies. *Virology* **4**, 328–345.

CARROLL, A. R. & WAGNER, R. R. (1979). Role of the membrane protein (M) in endogenous inhibition of *in vitro* transcription by vesicular stomatitis virus. *Journal of Virology* **29**, 134–142.

CATON, A. J. & ROBERTSON, J. S. (1980). Stucture of the 5′ ends of influenza virus mRNA produced *in vivo*. *Nucleic Acids Research* **8**, 2591–2603.

CHOW, L. T., BROKER, T. R. & LEWIS, J. B. (1979). Complex splicing patterns of RNAs from the early regions of adenovirus 2. *Journal of Molecular Biology* **134**, 265–303.

CLINTON, G. M., LITTLE, S. P., HAGEN, F. S. & HUANG, A. S. (1978). The matrix (M) protein of vesicular stomatitis virus regulates transcription. *Cell* **15**, 1455–1462.

FRANKLIN, R. M. & BREITENFELD, P. M. (1959). The abortive infection of Earle's L cells by fowl plague virus. *Virology* **8**, 293–307.

GANDHI, S. S., BELL, H. B. & BURKE, D. C. (1971). Abortive infection of L cells by fowl plague virus: comparison of RNA and protein synthesis in infected chick and L cells. *Journal of General Virology* **13**, 423–432.

HAY, A. J., ABRAHAM, G., SKEHEL, J. J., SMITH, J. C. & FELLNER, P. (1977*a*). Influenza virus messenger RNAs are incomplete transcripts of the genome RNAs. *Nucleic Acids Research* **4**, 4197–4209.

HAY, A. J., LOMNICZI, B., BELLAMY, A. R. & SKEHEL, J. J. (1977*b*). Transcription of the influenza virus genome. *Virology* **83**, 335–337.

INGLIS, S. C. (1978). *The synthesis of influenza virus proteins*. Ph.D. thesis, University of Cambridge, U.K.

INGLIS, S. C. & BROWN, C. M. (1981). Spliced and unspliced RNAs encoded by virion RNA segment 7 of influenza virus. *Nucleic Acids Research* **9**, 2727–2740.

INGLIS, S. C. & MAHY, B. W. J. (1979). Polypeptides specified by the influenza virus genome. III. Control of synthesis in infected cells. *Virology* **95**, 154–164.

INGLIS, S. C., CARROLL, A. R., LAMB, R. A. & MAHY, B. W. J. (1976). Polypeptides specified by the influenza virus genome. I. Evidence for eight distinct gene products specified by fowl plague virus. *Virology* **74**, 489–503.

INGLIS, S. C., McGEOCH, D. & MAHY, B. W. J. (1977). Polypeptides specified by the influenza virus genome. II. Assignment of protein coding functions to individual genome segments by *in vitro* translation. *Virology* **78**, 522–536.

INGLIS, S. C., CONTI, G. & MAHY, B. W. J. (1978). Control of influenza virus polypeptide synthesis. In *Negative Strand Viruses and the Host Cell*, pp. 239–248. Edited by B. W. J. Mahy & R. D. Barry. London: Academic Press.

INGLIS, S. C., BARRETT, T., BROWN, C. M. & ALMOND, J. W. (1979). The smallest genome RNA segment of influenza virus contains two genes that may overlap. *Proceedings of the National Academy of Sciences, U.S.A.* **76**, 3790–3794.

INGLIS, S. C., GETHING, M.-J. & BROWN, C. M. (1980). Relationship between the messenger RNAs transcribed from two overlapping genes of influenza virus. *Nucleic Acids Research* **8**, 3575–3589.

LAMB, R. A. & CHOPPIN, P. W. (1978). Effect of the host cell on early and late synthesis of influenza virus polypeptides. In *Negative Strand Viruses and the Host Cell*, pp. 229–237. Edited by B. W. J. Mahy & R. D. Barry. London: Academic Press.

LAMB, R. A. & CHOPPIN, P. W. (1981). Identification of a second protein (M_2) encoded by RNA segment 7 of influenza virus. *Virology* **112**, 729–737.

LAMB, R. A. & LAI, C.-J. (1980). Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. *Cell* **21**, 475–485.

LAMB, R. A. & LAI, C.-J. (1981). Conservation of the influenza virus membrane protein (M) amino acid sequence and an open reading frame of RNA segment 7 encoding a second protein (M_2) in H1N1 and H3N2 strains. *Virology* **112**, 746–751.

LAMB, R. A. & LAI, C.-J. (1982). Spliced and unspliced messenger RNAs synthesised from cloned influenza virus M DNA in an SV40 vector: expression of the influenza virus membrane protein (M_1). *Virology* **123**, 237–256.

LAMB, R. A., ETKIND, P. R. & CHOPPIN, P. W. (1978). Evidence for a ninth influenza viral polypeptide. *Virology* **91**, 60–78.

LAMB, R. A., LAI, C.-J. & CHOPPIN, P. W. (1981). Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proceedings of the National Academy of Sciences, U.S.A.* **78**, 4170–4174.

McCAULEY, J. W., MAHY, B. W. J. & INGLIS, S. C. (1982). Nucleotide sequence of fowl plague virus RNA segment 7. *Journal of General Virology* **58**, 211–215.

MAHY, B. W. J., CARROLL, A. R., BROWNSON, J. M. T. & McGEOCH, D. J. (1977). Block of influenza virus replication in cells preirradiated with ultra-violet light. *Virology* **83**, 150–161.

MINOR, P. D. & DIMMOCK, N. J. (1975). Inhibition of synthesis of influenza virus proteins: evidence for two host cell-dependent events during multiplication. *Virology* **67**, 114–123.

MINOR, P. D. & DIMMOCK, N. J. (1977). Selective inhibition of influenza virus protein synthesis by inhibitors of DNA function. *Virology* **78**, 393–406.

MOUNT, S. M. (1982). A catalogue of splice junctions. *Nucleic Acids Research* **10**, 459–472.

NEVINS, J. R. & WILSON, M. C. (1981). Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. *Nature, London* **290**, 113–118.

PORTER, A. G., SMITH, J. C. & EMTAGE, J. S. (1980). Nucleotide sequence of influenza virus RNA segment 8 indicates that the coding regions for NS_1 and NS_2 proteins overlap. *Proceedings of the National Academy of Sciences, U.S.A.* **77**, 5074–5078.

RITCHEY, M. B., PALESE, P. & SCHULMAN, J. L. (1976). Mapping of the influenza virus genome. III. Identification of genes coding for the nucleoprotein, membrane protein and nonstructural protein. *Journal of Virology* **20**, 307–313.

RODITI, I. (1983). *Cloning and sequence analysis of the influenza virus genome*. Ph.D. thesis, University of Cambridge, U.K.

SCHOLTISSEK, C., HARMS, E., ROHDE, W., ORLICH, M. & ROTT, R. (1976). Correlation between RNA fragments of FPV and their corresponding gene functions. *Virology* **74**, 332–344.

SKEHEL, J. J. (1973). Early polypeptide synthesis in influenza virus-infected cells. *Virology* **56**, 394–399.

SMITH, G. L. & HAY, A. J. (1982). Replication of the influenza virus genome. *Virology* **118**, 96–108.

VALCAVI, P., CONTI, G. & SCHITO, G. C. (1978). Macromolecular synthesis during abortive infection of KB cells by influenza virus. In *Negative Strand Viruses and the Host Cell*, pp. 475–481. Edited by B. W. J. Mahy & R. D. Barry. London: Academic Press.

WINTER, G. & FIELDS, S. (1980). Cloning of influenza cDNA into m13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. *Nucleic Acids Research* **8**, 1965–1974.

WINTER, G., FIELDS, S., GAIT, N. J. & BROWNLEE, G. G. (1981). The use of synthetic oligo-deoxyribonucleotide primers in cloning and sequencing segment 8 of influenza virus (A/PR/8/34). *Nucleic Acids Research* **9**, 237–245.

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