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Influence of the host cell on influenza virus replication

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[Plates 1 and 2]

The replication of influenza virus is characterized by a unique dependence upon host cell nuclear function. In contrast to all other negative strand RNA viruses, transcription from host cellular DNA is a prerequisite for the synthesis of virus-specific messenger RNA; new DNA synthesis is not required. We have analysed the distribution of each of the nine virus-specified proteins between the nucleus and cytoplasm of virus-infected cells, and find that in addition to the NP and the NS_1 proteins, two of the three P proteins show preferential migration into the nucleus. This subgroup of virus proteins may be involved in the early transcription of the viral genome which probably occurs in the nucleus. In non-permissive cell lines and in cells whose DNA function has been impaired by treatment with ultraviolet light, *N*-acetoxyacetaminofluorene or low doses of actinomycin D, production of some late virus proteins is inhibited. The specific host function required for this switch to late protein synthesis is unknown but in the cells treated with actinomycin D an abnormal accumulation of virus-specific mRNA occurs in the nucleus. In all cases studied, synthesis of new vRNA ceases when production of these late proteins has been blocked.

Introduction

Influenza virus replication differs in several important respects from that of other negative-stranded RNA viruses. Not only does the segmented nature of the genome raise unusual problems concerning regulation of virus RNA synthesis and virion assembly, but there is considerable evidence that virus-specific RNA synthesis requires participation of the host cell nucleus. This was originally suggested because of the inhibitory action on multiplication of influenza virus of actinomycin D (Barry *et al.* 1962), a drug that prevents DNA-dependent RNA transcription (Reich 1964). Subsequently it was shown that virus replication is also sensitive to several other drugs that cause damage to host cell DNA (Nayak & Rasmussen 1966; Rott *et al.* 1965) and to ultraviolet irradiation of the host cell before infection (Barry 1964; White *et al.* 1965). In contrast, inhibition of cell DNA synthesis, rather than of DNA function, has no inhibitory effect and may even enhance influenza virus multiplication (Scholtissek & Rott 1961; Brownson & Mahy 1979).

Crucial to our understanding of the function provided by host cell DNA was the discovery that the drug α-amanitin was a powerful inhibitor of early virus-specific RNA synthesis in influenza-infected cells (Mahy *et al.* 1972). This drug specifically inhibits cellular DNA-dependent RNA polymerase form II activity by binding to an enzyme subunit; the drug does not inhibit influenza virus replication in α-amanitin-resistant mutant cells that have an altered enzyme subunit (Lamb & Choppin 1977; Spooner & Barry 1977).

Recent work in our laboratory with the use of the avian influenza A virus, fowl plague, has

established the coding functions of each of the eight RNA segments that constitute the virus genome (table 1) as well as the 3′ and 5′ terminal nucleotide sequences (Robertson, this symposium). There are seven primary virion proteins and two proteins found only in infected cells (NS_1 and NS_2), which both appear to be encoded in virion RNA segment 8 (Inglis & Almond, this symposium). The amounts of each virus-specific protein synthesized in infected cells reflect the relative proportions of each gene transcript present in the cytoplasm at various times after

TABLE 1. RNA SEGMENTS AND PROTEINS OF INFLUENZA A (FOWL PLAGUE, ROSTOCK) VIRUS

genome RNA		coding capacity:	virus protein	
RNA no.	nucleotide chain length	maximum polypeptide chain length	polypeptide chain length	gene product
1	3500	1170	870	P2
2	3000	1000	960	P1
3	2950	980	850	P3
4	2450	820	750	haemagglutinin (HA)
5	2000	670	530	nucleoprotein (NP)
6	1720	570	450	neuraminidase (NA)
7	1080	310	250	matrix (M)
8	870	270	230	non-structural 1 (NS_1)
			110	non-structural 2 (NS_2)

The coding functions of each segment were determined as described by Almond & Barry (1979) and Inglis *et al.* (1977).

infection (Inglis *et al.* 1978). Control of influenza virus protein synthesis seems therefore to occur at the level of transcription, and there is evidence that during normal virus replication there are three distinct stages (Inglis & Mahy 1979):

1. A very early stage in which primary transcription of the infecting virus genome by the virion transcriptase results in synthesis of all species of virus mRNA except that coding for the NS_2 protein (primary transcription).

2. An early stage ($\frac{1}{2}$–2 h after infection) in which there is a specific increase in production of mRNA's coding for the NP and NS_1 proteins (early secondary transcription).

3. A late stage in which the mRNA coding for the M protein is predominant, and mRNAs coding for the HA, NA and NS_2 proteins increase in amount (late secondary transcription).

We are currently investigating the role of the host cell nucleus during influenza virus replication in the light of this new evidence concerning the numbers of virus genes and of virus-specific proteins, and the observed three stages during normal virus replication.

MIGRATION OF VIRUS-SPECIFIC PROTEINS INTO THE CELL NUCLEUS

The first experimental observation suggesting that the cell nucleus is involved in influenza virus replication was made by Watson & Coons (1954) who monitored the appearance of influenza virus antigens in the amniotic cells of fertile eggs by using a fluorescein-conjugated antibody prepared against the total structural components of the virus. Fluorescence developed initially in the cell nucleus alone, and subsequently throughout the cell. The same effect was demonstrated by Liu (1955) in ferret respiratory tract cells infected with influenza virus; in addition he used a fluorescent antiserum absorbed with excess NP antigen to show that the

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nuclear staining was due to NP. Similar studies were reported by Breitenfeld & Schafer (1957) and Maeno & Kilbourne (1970) for tissue culture cells infected by influenza virus.

These observations with the use of fluorescent antibody were confirmed by polyacrylamide gel electrophoresis of radioactively labelled polypeptides in cells infected with influenza virus, and in pulse–chase experiments it was found that in addition to NP, the NS_1 polypeptide migrated into the nucleus of infected cells (Lazarowitz *et al.* 1971; Taylor *et al.* 1969, 1970; White *et al.* 1970). A non-structural protein, probably NS_1, was also found by fluorescent antibody staining to accumulate in the infected cell nucleolus (Dimmock & Watson 1970). Of the other seven virus-induced polypeptides, only M (Gregoriades 1978; Hudson *et al.* 1978) and NS_2 (Krug & Etkind 1973) have been reported to enter the nucleus of infected cells.

We have re-examined this question by pulse–chase analysis of MDCK cells infected with influenza A virus (fowl plague, Rostock strain). This permissive cell line was chosen in preference to chick embryo fibroblasts because the MDCK cells are larger, the nuclei are more easily purified, and the yield of nuclear material is greater. Several nuclear purification techniques were compared. Each consisted of treating the cells with a combination of detergents for 30 min in hypotonic buffer, mixing, pelleting the nuclei by gentle centrifugation and then analysing the resulting cell fractions by polyacrylamide gel electrophoresis. The initial treatment tried was 0.5% Nonidet P40 alone. This resulted in a nuclear fraction containing large amounts of both HA and M proteins. Addition of deoxycholic acid (2 g/l) to the purification mix eliminated almost all nuclear HA, while addition of 0.5% Triton X100 (by volume) eliminated almost all nuclear M. The initial association of these proteins with nuclear fractions probably represented cytoplasmic contamination which was removed by more vigorous detergent treatment. Figure 1*a* shows the results of an experiment with cell fractions obtained after simultaneous treatment with all three of the detergents mentioned. The cells were pulse-labelled for 5 min with [^{35}S]methionine 4 h after infection with virus and then chased by removal of the radioactive medium and replacement with medium containing unlabelled methionine for the intervals stated on the figure. The gel (175 g/l) pattern shows that NP and NS_1 are the predominant viral polypeptides entering the nucleus, while NS_2, M and HA remain almost entirely cytoplasmic. The precursor HA_0 molecule can be seen to cleave with time into its daughter fragments HA_1 and HA_2 in the cytoplasm. In figure 1*b* a heavier exposure of a lower percentage gel separates all three P proteins. There is a clear association of all three P proteins with the nuclear fraction. However, relative to the amounts of each present in the cytoplasm, P1 and P2 seem to exhibit more migration into the nucleus than does P3. This may indicate that P3 plays a role in viral RNA synthesis different from that of the other two P proteins.

Further support for this idea was obtained by similar experiments carried out with the Dobson strain of fowl plague virus, in which the P2 protein is the functional equivalent of the P3 protein of the Rostock strain (Almond & Barry 1979). Pulse–chase analysis of MDCK cells infected with fowl plague virus Dobson showed that the nuclear fraction contained much more of proteins P1 and P3 than of protein P2 (data not shown). Migration of the NP and NS_1 proteins into the cell nucleus occurred with the Dobson as with the Rostock strain, and no evidence could be found to support previous claims that proteins M and NS_2 migrate into the nucleus (Gregoriades 1978; Hudson *et al.* 1978; Krug & Etkind 1973).

Effects of inhibitors of cell DNA function on influenza virus replication

We have previously reported that early influenza virus-specific RNA synthesis is completely inhibited in cells treated with α-amanitin (20 μg/ml) (Mahy *et al.* 1972) or with a high concentration (5 μg/ml) of actinomycin D (Mahy *et al.* 1977). It seems likely that in such cells primary virus RNA transcription is abolished owing to inhibition of the synthesis of cellular mRNA required as a primer for the virion transcriptase (Krug *et al.*, this symposium). Other treatments that inhibit cell DNA function, such as preirradiation of the host cells with ultraviolet light, block virus replication completely by preventing late protein synthesis without affecting early protein synthesis (Mahy *et al.* 1977). Ultraviolet irradiation causes pyrimidine dimer formation which blocks DNA transcription.

We have recently compared the effects on influenza virus-specific protein synthesis of preirradiation of the host cell with ultraviolet light (36 J/m^2), or preincubation with the addition of 200 μM *N*-acetoxyacetylaminofluorene (AAAF) to the cell medium. The latter drug prevents DNA transcription by causing substitution of C-8 of purines with the bulky acetylaminofluorene group (Cerutti 1975). Similar effects on virus-specific protein synthesis are seen with each inhibitor (figure 2). Synthesis of early virus-specific proteins (P1–3, NP, NS_1) is scarcely affected, but late protein synthesis (HA, M) is abolished. An interesting exception is the late protein NS_2, which is synthesized in similar amounts in normal as in inhibited cells. This might be related to the fact that it is synthesized, like the early protein NS_1, from a transcript of gene 8 (Inglis & Almond, this symposium).

Synthesis of influenza virus-specific proteins in a non-permissive host cell

We have found that a similar inhibition of late, but not early, virus-specific protein synthesis occurs in L-cells infected with influenza A (fowl plague) virus. This virus–cell system has long been known to be abortive for fowl plague virus replication and in particular it was reported that the 'soluble antigen' (RNP) failed to migrate from the nucleus of infected L-cells as it did in permissive chick embryo fibroblast cells (Franklin & Breitenfeld 1959). We have used two strains of fowl plague virus, a cloned isolate (S3) of the Rostock strain, and a cloned host-range mutant (4H) of the Dobson strain, which was originally selected by Zavada for its ability to plaque in BHK-21 cells, but which will also plaque in L-cells (Almond & Barry 1978).

Description of plate 1

Figure 1. (*a*) Protein patterns in subcellular fractions of fowl plague virus Rostock infected MDCK cells: 4 h after infection, cells were pulse-labelled with [^{35}S]methionine for 5 min, the labelled medium was removed, and the labelled proteins were then chased for increasing lengths of time by addition of medium containing unlabelled methionine. U, uninfected; numbers indicate lengths of chase in minutes. Polyacrylamide gel (175 g/l). Photographic film exposed for 1 week.

(*b*) Detail of polyacrylamide gel (70 g/l) of the same specimens as shown in (*a*) after 3 weeks of exposure of the photographic film.

Figure 2. Time course of protein synthesis in fowl plague virus Rostock infected chick embryo fibroblasts after no pretreatment (C), preirradiation with 36 J/m^2 ultraviolet light (UV), or preincubation in 200 μM *N*-acetoxyacetylaminofluorene (AAAF). U, uninfected; numbers indicate time in hours after infection. Polyacrylamide gel (175 g/l).

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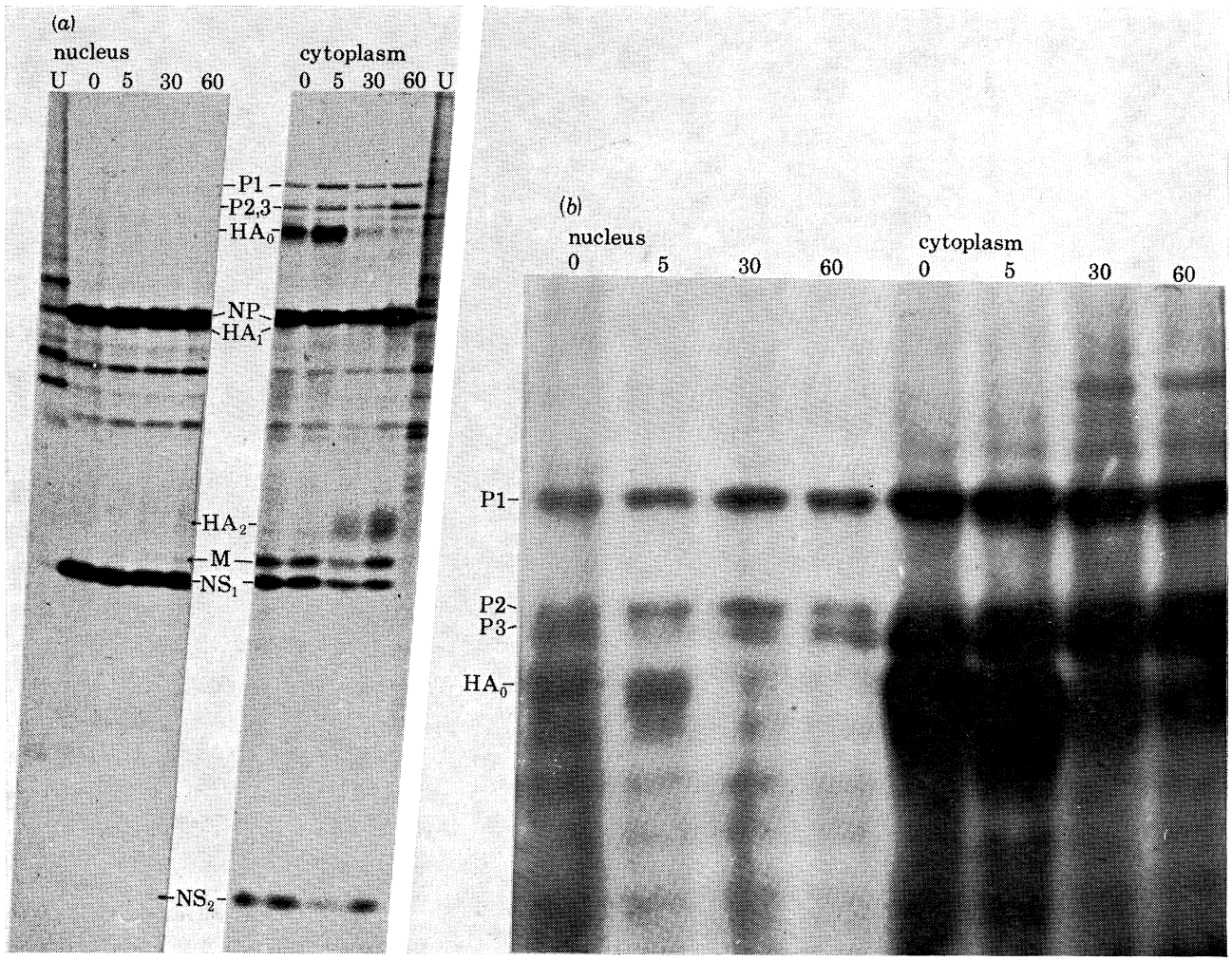

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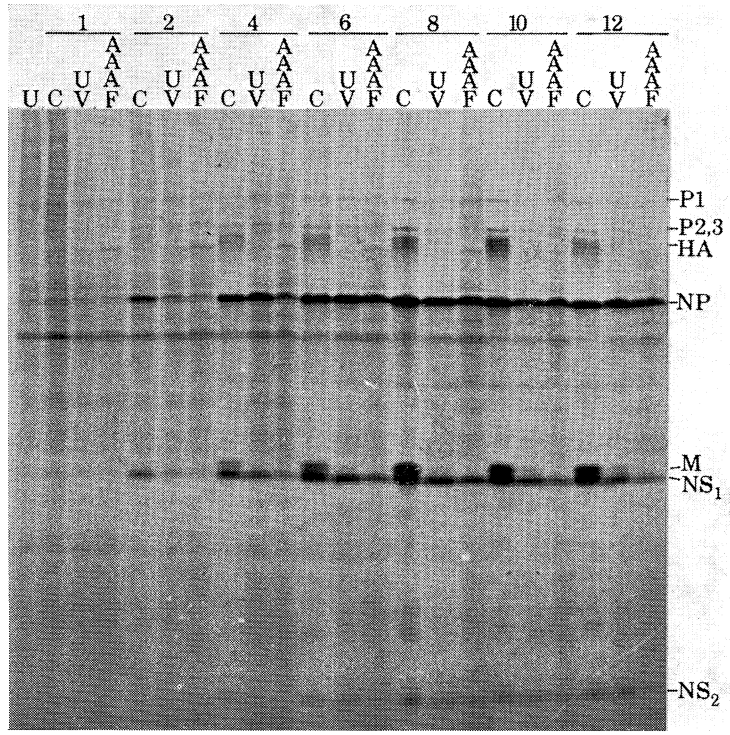

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FIGURES 1 AND 2. For description see opposite.

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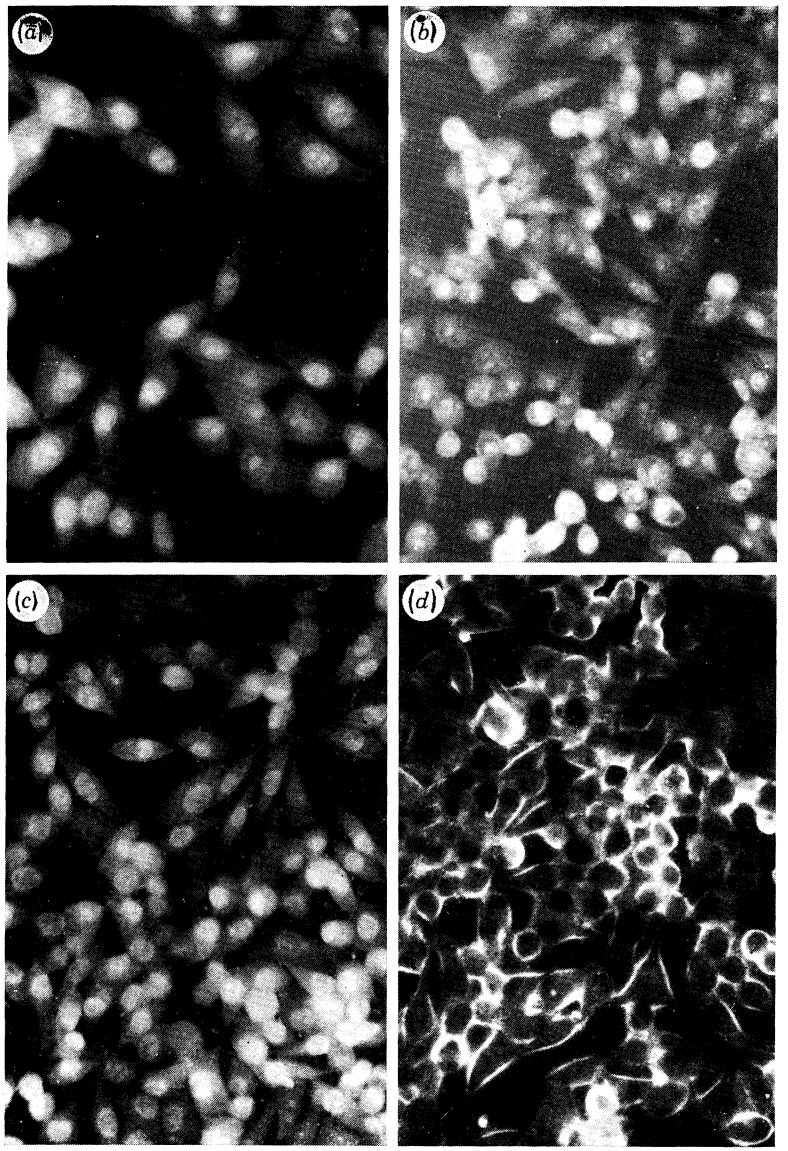

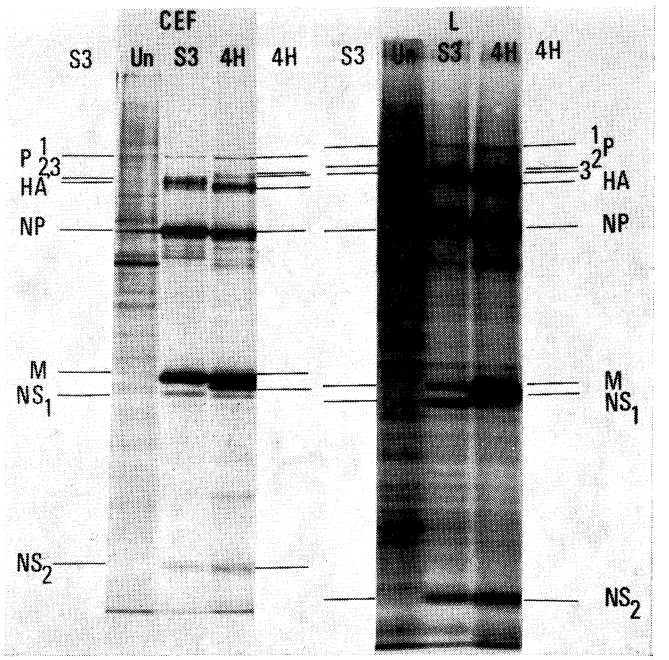

FIGURE 4. Immunofluorescence of fowl plague virus-infected L-cell cultures detected with an antiserum to fowl plague virus ribonucleoprotein antigen. (*a*) Rostock strain (S3) 2 h after infection; (*b*) Dobson strain (4H) 2 h after infection; (*c*) Rostock strain (S3) 4 h after infection; (*d*) Dobson strain (4H) 4 h after infection.

FIGURE 5. Polyacrylamide gel electrophoresis of proteins synthesized in CEF cells or L-cells infected with Rostock (S3) or Dobson (4H) strains of fowl plague virus or uninfected (Un). Cells were pulse-labelled with [^{35}S]methionine (5 μCi/ml) for 15 min at 4 h after infection and polypeptides separated on a polyacrylamide gel (175 g/l).

The yields of these two viruses were similar in chick embryo fibroblast cells; although the yields of both viruses were reduced in L-cells, there was a tenfold greater yield of cell-associated infectious 4H virus (figure 3). The distribution of ribonucleoprotein antigen was studied in L-cells at 2 and 4 h after infection. The antigen remained predominantly intranuclear in cells infected with the S3 virus, but had migrated out of the nucleus by 4 h after infection with the

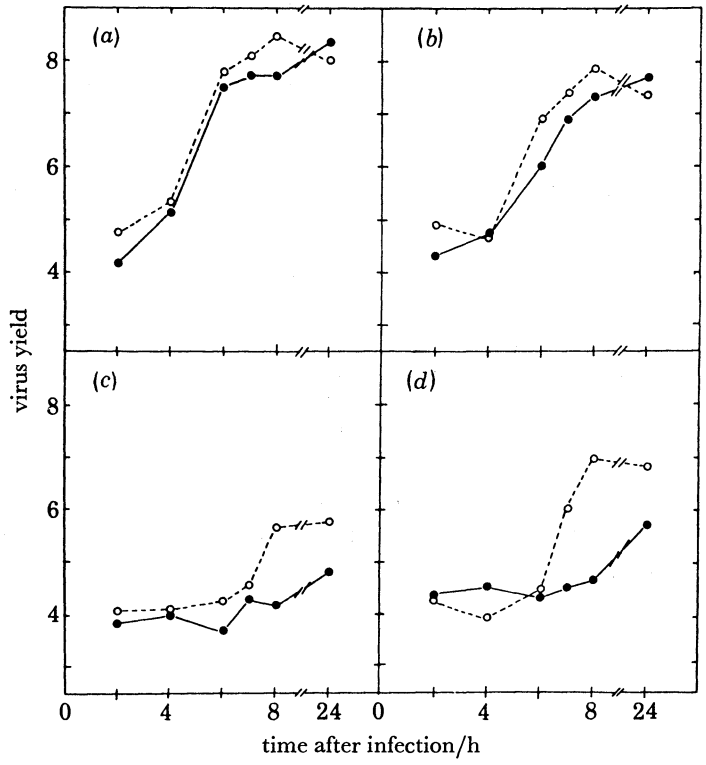

FIGURE 3. Growth curves of (*a*) fowl plague virus Rostock in CEF cells; (*b*) fowl plague virus Dobson in CEF cells; (*c*) fowl plague virus Rostock in L-cells; (*d*) fowl plague virus Dobson in L-cells. ●, Released infectious virus; ○, cell-associated infectious virus. Virus yield is expressed as the decadic logarithm of plaque-forming units per culture.

mammalian cell-adapted 4H virus (figure 4, plate 1). The synthesis of virus-specific polypeptides in each cell type was studied by pulse-labelling with [^{35}S]methionine 4 h after infection with S3 or 4H virus (figure 5). In permissive CEF cells infected with S3 virus there was a much greater synthesis of M relative to NS_1 polypeptides; this situation was reversed in the non-permissive L-cells. Polypeptide synthesis in response to infection with the 4H virus was closely similar in the two cell types.

ACTINOMYCIN D AT LOW CONCENTRATION ALTERS TRANSPORT OF mRNAs FROM NUCLEUS TO CYTOPLASM

Inhibition of the synthesis of influenza virus M protein also occurs during infection of CEF cells treated with 0.1 μg/ml actinomycin D; previous studies indicated that this was due, as with irradiation with ultraviolet, to a specific decrease in the amount of mRNA coding for M protein (Inglis *et al.* 1978; Inglis & Mahy 1979). Recently we have used highly sensitive

hybridization methods to analyse the amounts of mRNA coding for various influenza virus polypeptides (Barrett *et al.* 1978, 1979).

In cells treated with actinomycin D (0.1 μg/ml) there was an overall reduction in the rate of virus-specific cRNA synthesis, but up to 2 h after infection all the cRNAs accumulated in the cytoplasm. However, between 2 and 4 h after infection, when amplification of synthesis of M protein mRNA (late secondary transcription) normally occurs, there was an accumulation of all virus-specific cRNAs in the nucleus and no further increase in the cytoplasm (table 2). Analysis of individual mRNAs showed that by 4 h after infection the amount of mRNA coding for M protein was ten times greater in the nucleus than in the cytoplasm (Barrett *et al.* 1979).

TABLE 2. ESTIMATED NUMBER OF POLYADENYLATED cRNA GENOME COPIES IN THE NUCLEUS AND CYTOPLASM OF ACTINOMYCIN D-TREATED CELLS AT VARIOUS TIMES AFTER INFECTION

time after infection/h ...	1	2	4
cytoplasm	5	28	28
nucleus	7	12	252

Chick embryo fibroblast cell monolayer cultures were pretreated at 37 °C for 1 h with medium containing actinomycin D (0.1 μg/ml) and infected in the presence of the drug and polyadenylated RNA extracted as described in detail by Barrett *et al.* (1979). The number of gene copies per cell nucleus or cytoplasm was determined by hybridization with ^{125}I-labelled influenza virion RNA (Barrett *et al.* 1979).

TABLE 3. ESTIMATED NUMBER OF NON-POLYADENYLATED cRNA AND vRNA GENOME COPIES IN THE NUCLEUS AND CYTOPLASM OF UNTREATED AND ACTINOMYCIN D-TREATED CELLS AT VARIOUS TIMES AFTER INFECTION

time after infection/h ...	1		2		4	
fraction	cRNA	vRNA	cRNA	vRNA	cRNA	vRNA
nucleus						
+ act. D	70	25	160	60	200	50
− act. D	100	35	140	60	440	520
cytoplasm						
+ act. D	240	60	300	60	400	150
− act. D	90	20	1400	170	1800	660
total						
+ act. D	310	85	460	120	600	200
− act. D	190	55	1540	230	2240	1180

Chick embryo fibroblast cell monolayer cultures were pretreated at 37 °C for 1 h with medium containing actinomycin D (0.1 μg/ml) and infected in the presence of the drug. The number of cRNA and vRNA copies of the virus genome present in the non-polyadenylated RNA extracted from the cells was determined by hybridization with ^{125}I-labelled influenza vRNA and a ^{3}H-cDNA copy of vRNA. Detailed methods are described by Barrett *et al.* (1979).

We also measured the amounts of vRNA and non-polyadenylated cRNA (template RNA) in these actinomycin D-treated infected cells (table 3). The amount of virus-specific template RNA present in actinomycin D-treated cells was similar to that in control cells at 1 h after infection, but there was a 3–4-fold reduction at later times, particularly in the cytoplasmic fractions. The accumulation of vRNA was similar in drug-treated and normal cells up to 2 h after infection, but the marked increase in the amounts of vRNA was abolished in both the nucleus and cytoplasm of cells treated with actinomycin D.

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It is not certain what effect this concentration of actinomycin D has on cell metabolism, but there is evidence that nucleolar function may undergo the greatest inhibition (Rickinson & Dendy 1969) since the nucleolus is GC-rich, and actinomycin D specifically intercalates between G–C base pairs in DNA. The nucleolus has been implicated in the transport of messenger RNAs from the nucleus to the cytoplasm (Deak *et al.* 1972). Our results taken together imply that a cell function sensitive to actinomycin D (0.1 μg/ml) or preirradiation with ultraviolet light is necessary at about 2 h after infection in cells infected with influenza virus. Inhibition of this function results not only in hold-up of virus-specific mRNA in the nucleus and cessation of vRNA synthesis, but also specific reduction in the synthesis of all late virus specific proteins except NS_2.

Conclusions

Several stages in the replication of influenza virus require the participation of the host cell (table 4). Early in infection, the first stage that can be detected is primary transcription of the incoming virus RNA which is unselective and so results in the synthesis of small amounts of all the virus-specific proteins except NS_2 (Inglis & Mahy 1979). This primary transcription stage apparently requires a host cell-coded mRNA as part of the transcription complex (Krug *et al.*, this symposium) and can be blocked by α-amanitin or high concentrations of actinomycin D (Mahy *et al.* 1972, 1977). The second stage, early secondary transcription, involves modification of the transcription process by a product or products of the translation of primary transcripts, so that relatively large amounts of mRNAs coding for NP and NS_1 are synthesized. This early secondary transcription does not occur if protein synthesis is blocked, for example by cycloheximide (Inglis & Mahy 1979).

Table 4. Inhibitors of influenza virus replication

replication stage	*inhibitor*	*function inhibited*
primary transcription	α-amanitin	host cell RNA polymerase II; synthesis of host cell RNA primer
	actinomycin D (> 5 μg/ml)	
early secondary transcription	cycloheximide (100 μg/ml)	translation of primary transcripts
late secondary transcription and vRNA synthesis	actinomycin D (0.1 μg/ml)	?
	N-acetoxyacetylaminofluorene (200 μM)	
	u.v. irradiation before infection	
	non-permissive infection	

During this stage of the replication cycle large amounts of the NP and NS_1 proteins, as well as two of the P proteins (P1 and P2 for fowl plague virus, Rostock), accumulate in the cell nuclear fraction. Studies with temperature sensitive mutants implicate the NP and P proteins in the process of transcription (Barry & Mahy 1979) and accumulation of these proteins in the nucleus may be related to the fact that the nucleus is probably the site at which early transcription occurs. The majority of virus-specific mRNA can be detected in the nucleus early in infection (Barrett *et al.* 1978).

The third stage in virus replication involves late secondary transcription and new virion RNA synthesis. During this late stage there is amplification of the transcription of those mRNAs

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coding for the HA, NA, M and NS_2 proteins, and a resultant increase in late protein synthesis so that M, the most abundant virion protein (Inglis *et al.* 1976), becomes the major virus-specific translation product. This late stage is blocked in certain non-permissive host cells and also by a variety of agents that damage the function of DNA (table 4). There is evidence that this block is associated with a specific decrease in the concentration of late protein mRNAs in the non-permissive infection of L-cells (Bosch *et al.* 1978), and pretreatment of permissive host cells with ultraviolet irradiation (Mahy *et al.* 1977) or 0.1 μg/ml actinomycin D (Barrett *et al.* 1979). Curiously, none of these inhibitors affect the mRNA for the NS_2 protein, the function of which is currently unknown. However, in all cases we have studied, the synthesis of new virion RNA ceases when late secondary transcription is blocked.

The nature of the host function which is necessary for late secondary transcription is unknown, but Minor & Dimmock (1975, 1977) have suggested, on the basis of inhibitor studies, that it may involve a nucleolar function mediated by NS_1, which is known to accumulate in the nucleolus (Dimmock & Watson 1970). Our evidence does not conflict with a role for the nucleolus in late secondary transcription, and in particular the accumulation of transcripts within the nucleus in cells treated with low concentrations of actinomycin D (table 2) supports the idea that the nucleolus may provide an essential transport function. However, recent work in our laboratory with the use of viruses with a temperature-sensitive mutation in gene 8 has shown that although replication is blocked, at the restrictive temperature, at the stage of late secondary transcription, virus specific mRNA is not held up in the nucleus. Further work is clearly necessary to establish the true influence of the host cell on virus-specific late secondary transcription.

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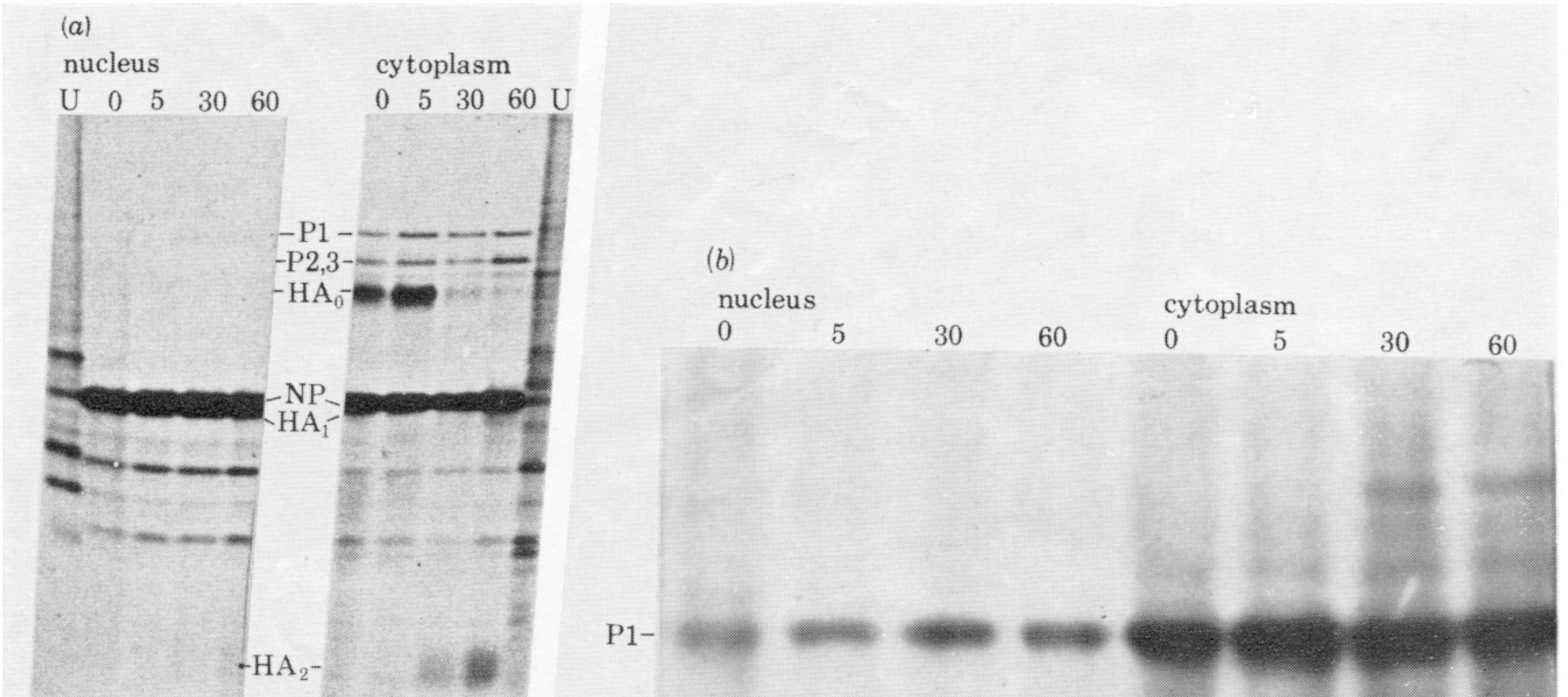

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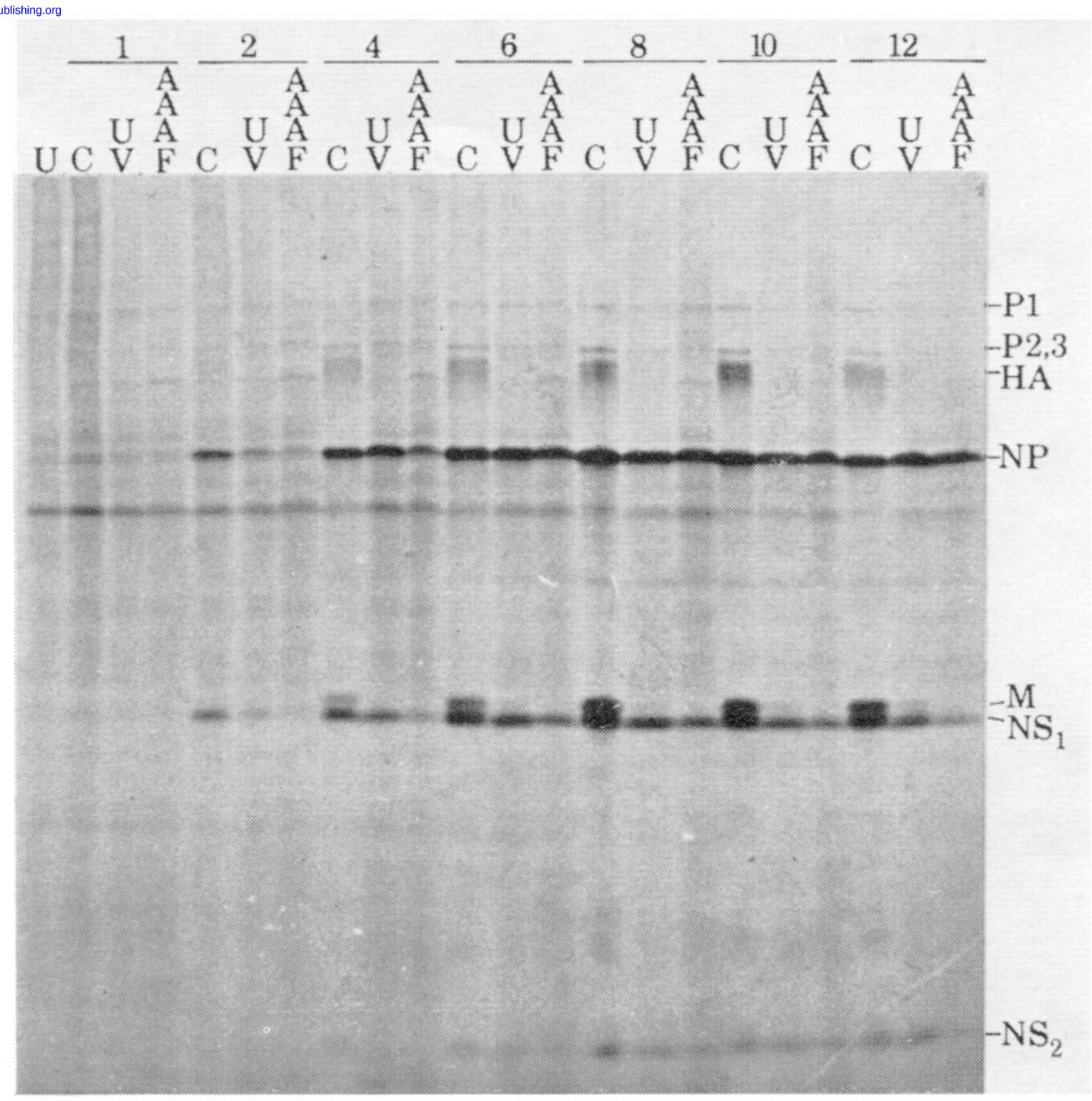
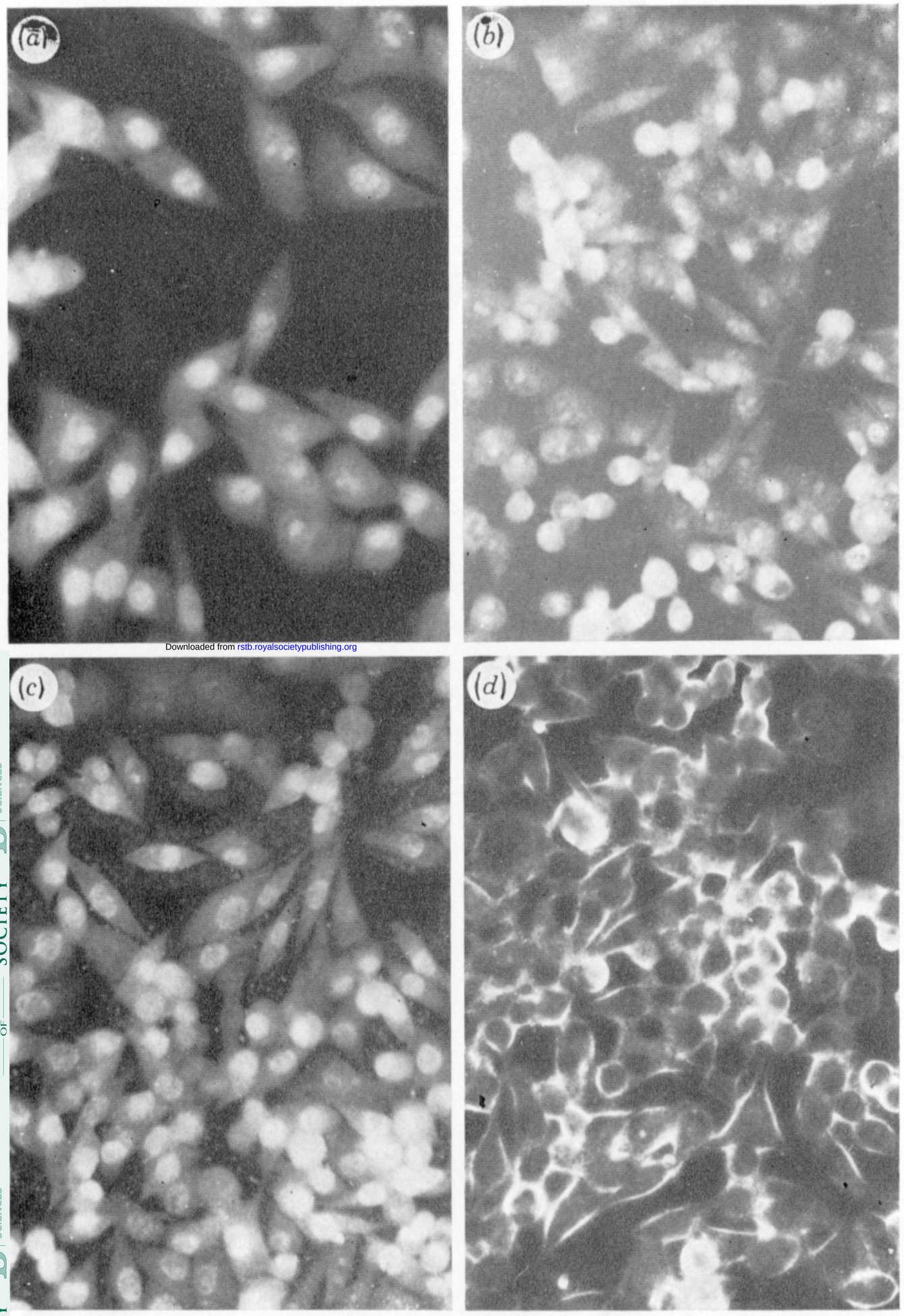


2

FIGURES 1 AND 2. For description see opposite.

4



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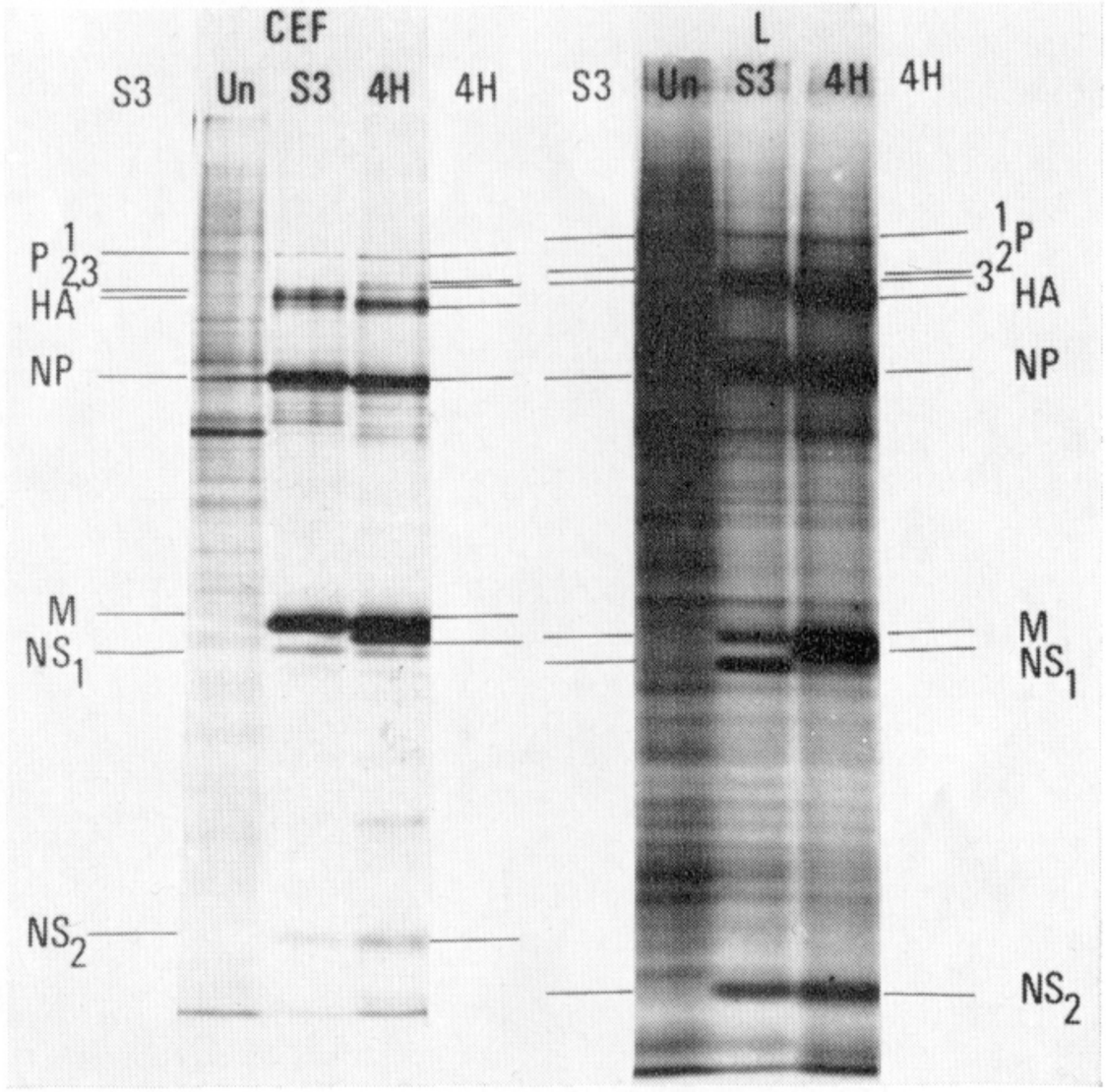

FIGURE 4. Immunofluorescence of fowl plague virus-infected L-cell cultures detected with an antiserum to fowl plague virus ribonucleoprotein antigen. (*a*) Rostock strain (S3) 2 h after infection; (*b*) Dobson strain (4H) 2 h after infection; (*c*) Rostock strain (S3) 4 h after infection; (*d*) Dobson strain (4H) 4 h after infection.

FIGURE 5. Polyacrylamide gel electrophoresis of proteins synthesized in CEF cells or L-cells infected with Rostock (S3) or Dobson (4H) strains of fowl plague virus or uninfected (Un). Cells were pulse-labelled with [^{35}S]methionine (5 μCi/ml) for 15 min at 4 h after infection and polypeptides separated on a polyacrylamide gel (175 g/l).

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