PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

A Ninth Unique Influenza Virus-Coded Polypeptide

R. A. Lamb and P. W. Choppin

Phil. Trans. R. Soc. Lond. B 1980 **288**, 327-333
doi: 10.1098/rstb.1980.0008

Phil. Trans. R. Soc. Lond. B **288**, 327–333 (1980)
Printed in Great Britain

A ninth unique influenza virus-coded polypeptide

By R. A. Lamb and P. W. Choppin
The Rockefeller University, New York, New York 10021, *U.S.A.*

A polypeptide with a molecular mass of *ca.* 11000 has been found in various cell types infected with several strains of influenza A and B viruses. Differences have been found in the electrophoretic mobility of this polypeptide in cells infected with different strains, suggesting that it is virus coded. Peptide mapping studies have shown that this polypeptide is distinct from the eight defined influenza virus gene products. A fraction containing a virus-specific mRNA with an estimated size of *ca.* 300–400 nucleotides has been obtained. Schemes for the synthesis of this mRNA are discussed.

Introduction

Influenza virus contains eight discreet pieces of negative-stranded RNA which are the genes that code for the seven viral structural proteins, i.e. three polymerase-associated proteins (P1, P2 and P3), the haemagglutinin (HA), the neuraminidase (NA), the nucleocapsid protein (NP) and the membrane protein (M), and for a non-structural protein (NS) found only in infected cells. Assignment of the genes coding for the polypeptides has been made by several methods (reviewed in Barry & Mahy 1979). In addition to the eight virus-specific polypeptides found in influenza virus infected cells and described above, four small polypeptides have been observed, designated 1–4 (Lamb & Choppin 1978; Lamb *et al.* 1978). Polypeptides 1, 2 and 3 (molecular masses *ca.* 22000, 19000 and 17000 respectively), have been shown to be related to NS by tryptic peptide mapping (Lamb *et al.* 1978). Polypeptide 4 (molecular mass *ca.* 11000) is thought to be the same polypeptide observed in other studies and designated 9 or NS_2 (Skehel 1972; Follett *et al.* 1974; Etkind & Krug 1975; Minor & Dimmock 1975, 1977). The experiments described briefly here were carried out to clarify the nature of this polypeptide.

Materials and methods

The virus, cells and procedures used have been described in detail previously (Lamb & Choppin 1977*a*, *b*; Lamb *et al.* 1978). Any modifications of these procedures are indicated in the figure legends.

Results

In a wide variety of cell types, the influenza virus specific polypeptides can be detected above the host cell background (Lazarowitz *et al.* 1971; Skehel 1972; Inglis *et al.* 1976; Lamb & Choppin 1976; Lamb *et al.* 1978). As shown in figure 1, in cells infected with the WSN strain of influenza A virus a polypeptide (4)† of molecular mass *ca.* 11000 is synthesized, in addition to eight defined influenza virus gene products. This has been found in chick embryo fibroblasts

† The designation 4 follows the terminology used previously (Lamb & Choppin 1978; Lamb *et al.* 1978). However, in view of the mounting evidence, including that presented here, that it is a unique virus-coded protein, NS_2 would appear to be the appropriate designation.

(CEF), L cells, African green monkey kidney cells (CV-1), Chinese hamster ovary cells (CHO-S), HeLa cells, MDBK cells, and several other cell types. The amount of polypeptide 4 synthesized varied among infected cell types, but this could not be correlated with the ability of the cells to produce infectious virions. It has been found in this laboratory that infection with paramyxoviruses may enhance the synthesis of certain host cell polypeptides (Peluso *et al.* 1977, 1978). That polypeptide 4 was a virus-specific polypeptide and not a host polypeptide induced by virus infection was shown by infecting the same host cell (HeLa cells) with a variety of strains of influenza virus and searching for strain-specified differences. Polypeptide 4 was found in each case, and there were differences in the mobility of the polypeptide depending on the strain (figure 2). The resistance of synthesis of polypeptide 4 to high salt treatment of infected cells has also suggested the viral specificity of this polypeptide (Lamb *et al.* 1978). Experiments designed to investigate the intracellular location of polypeptide 4 by fractionation of infected cells into cytoplasm and nucleus have shown that 4 is predominantly cytoplasmic (results not shown).

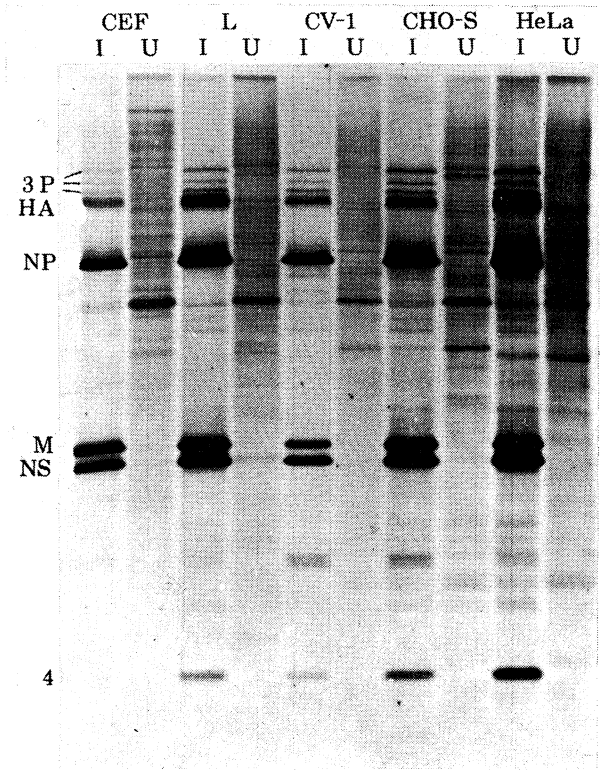

FIGURE 1. The synthesis of polypeptide 4 in WSN infected CEF, L, CV-1, CHO-S and HeLa cells. I, infected cells; U, uninfected cells (Lamb *et al.* 1978).

Influenza B virions have a structure similar to that of influenza A virions, and have been shown to contain similar polypeptides and eight RNA segments (Oxford 1973; Choppin *et al.* 1975; Racaniello & Palese 1979). HeLa cells were infected with three influenza B strains and examined for the synthesis of a small polypeptide analgous to 4. As illustrated in figure 3 with the Lee strain a polypeptide with a molecular mass of *ca.* 11000 was identified.

To investigate whether polypeptide 4 in influenza virus-infected cells was distinct from the other viral polypeptides, the tryptic peptides of all the virus-specific polypeptides of the WSN

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

strain of influenza A were compared on cellulose plates by using a highly reproducible system of high voltage electrophoresis and chromatography (Lamb *et al.* 1978), and the results suggested that polypeptide 4 was unique. In these experiments polypeptide NA was compared only indirectly with polypeptide 4; however, we have recently obtained tryptic peptide maps of NA isolated from purified virions by Triton X-100 fractionation (Scheid *et al.* 1972) and purified by polyacrylamide gel electrophoresis, and these have shown directly that polypeptide 4 is not related to NA. Thus, the results indicate that polypeptide 4 is unique, and not derived from one of the eight defined influenza virus polypeptides.

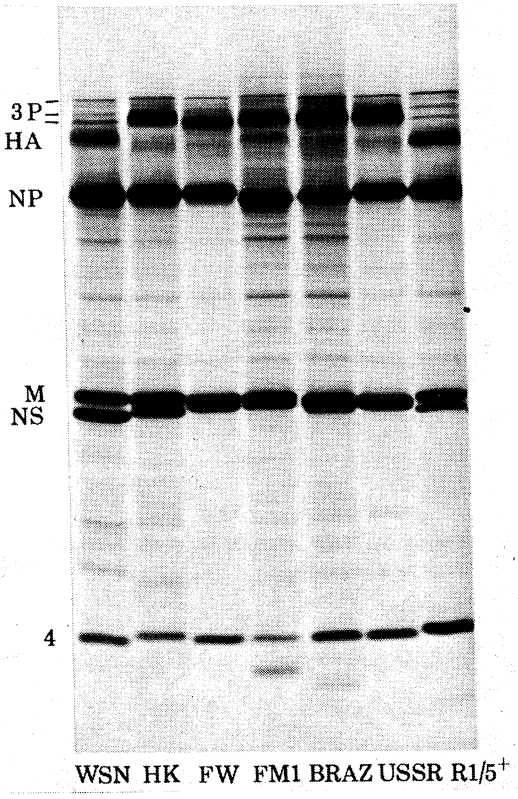

FIGURE 2

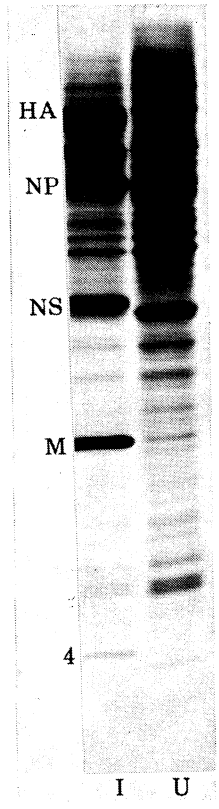

FIGURE 3

FIGURE 2. The synthesis of polypeptide 4 in HeLa cells infected with various strains of influenza A virus. At 6 h after infection, cells were labelled with [^{35}S]methionine (20 μCi/ml) for 30 min and subjected to electrophoresis on a 17.5% acrylamide, 4 M urea gel as described previously (Lamb *et al.* 1978). The strains used were, from left to right, WSN/33 (H0N1); Hong Kong/68 (H3N2); Fort Warren/50 (H1N1); FM1/47 (H1N1); Brazil/78 (H1N1); USSR/77 (H1N1); RI/5$^+$/57 (H2N2).

FIGURE 3. The synthesis of a polypeptide (4) of molecular mass *ca.* 11 000 in influenza B/Lee infected HeLa cells. At 6 h after infection cells were labelled with [^{35}S]methionine (20 μCi/ml) for 30 min and subjected to electrophoresis on a 17.5% acrylamide, 4 M urea gel as described previously (Lamb *et al.* 1978).

In infected cells the synthesis of polypeptide 4 was found to occur at a rate different from that of other influenza virus-specific polypeptides, in that it was detected late, i.e. 2–3 h after infection (Lamb *et al.* 1978). This suggested that the mRNA for the polypeptide was not expressed early in infection. To investigate this further, in-vitro translation was done with mRNAs extracted from infected cells treated continuously with cycloheximide to restrict RNA synthesis to that produced by the input virus transcriptase (Lam *et al.* 1978). These experiments

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

showed that all the viral polypeptides were synthesized except polypeptide 4. In other experiments designed to restrict protein synthesis to that directed by mRNA species synthesized by primary transcription, infected cells were treated with amino acid analogues, because the polypeptides including transcriptase molecules synthesized in their presence are likely to be non-functional. Under these conditions again all the virus-specific polypeptides except polypeptide 4 were synthesized. Finally, polypeptide 4 was not synthesized in an in-vitro coupled transcription–translation system (Lamb *et al.* 1978). These results suggested that the *ca.* 11000 molecular mass polypeptide 4 is a 'late' gene product whose synthesis is dependent on 'early' viral protein synthesis.

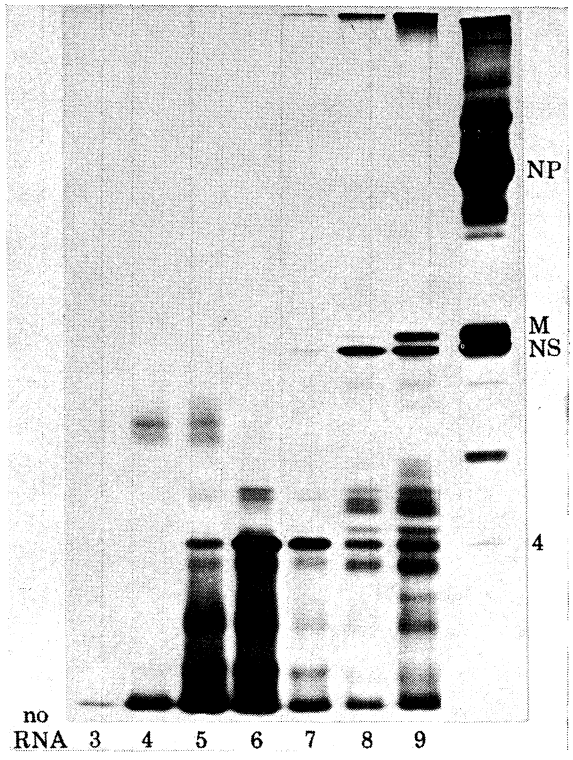

FIGURE 4. In-vitro translation of mRNAs from infected cells separated on sucrose density gradients. Poly(A)-containing RNA from the cytoplasm of WSN-infected CEF cells isolated 3 h after infection was dissolved in NETS buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 5 g/l SDS). The RNA was centrifuged for 20 h at 24000 rev/min at 22 °C in a 15–30 % (by mass) sucrose–NETS gradient in a Spinco SW27 rotor. Thirty fractions were collected and the RNA in each fraction translated in wheat germ extracts as described previously (Lamb *et al.* 1978). No RNA, no added RNA to wheat germ extract; lanes 3–9, translation of fractions 3–9. The far right lane contains WSN-infected CEF cell lysate to show viral polypeptides as markers.

Evidence for a specific mRNA for polypeptide 4 has been obtained by the separation of polyadenylated mRNAs from infected cells on sucrose density gradients and in-vitro translation of the fractions obtained. As shown in figure 4 (lanes 5 and 6) a polyadenylated mRNA with an estimated size of *ca.* 300–400 nucleotides can be translated to yield a polypeptide of molecular mass 11000, which tryptic peptide maps have shown to be similar to polypeptide 4 from infected cells. When this mRNA fraction was hybridized to virion RNA, high levels of hybridization were obtained, suggesting that it is a transcript from one of the eight virion segments.

Examination of purified influenza virions on polyacrylamide gels has revealed that very small amounts of a polypeptide of molecular mass *ca.* 11000 are present in the virion (figure 5,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

lane 2), and peptide mapping has indicated that this polypeptide is the same as polypeptide 4 found in infected cells (Lamb *et al.* 1978). The polypeptide of molecular mass *ca.* 11000 in virions is resistant to proteolytic cleavage by bromelain when intact virions are treated with the enzyme, suggesting that it resides within the lipid bilayer. Immunoprecipitation of infected HeLa cells with antisera prepared against purified virions precipitated polypeptide 4 (figure 5, lane 5) as did antiserum against an extract of infected MDCK cells (figure 5, lane 4).

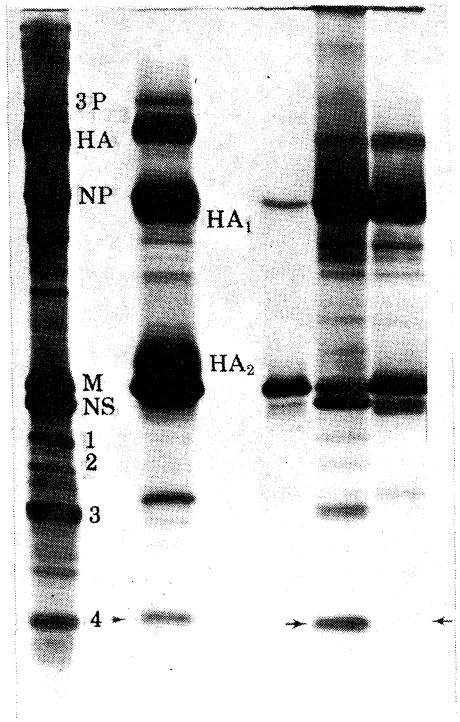

FIGURE 5. Immunoprecipitation of [^{35}S]methionine-labelled polypeptides from lysates of HeLa cells infected with the WSN strain of influenza A virus with antisera prepared against M protein, infected MDCK cells and virions, respectively. Lane 1, infected HeLa cell lysate, no antiserum; lane 2, purified marker virus; lane 3, cell lysates precipitated with antiserum to M; lane 4, cell lysate precipitated with antiserum to MDCK cells; lane 5, cell lysate precipitated with antiserum to purified virions (Lamb *et al.* 1978).

DISCUSSION

A polypeptide of molecular mass *ca.* 11000 designated 9, NS or 4 has previously been observed in influenza virus infected cells (Skehel 1972; Follet *et al.* 1974; Etkind & Krug 1975; Minor & Dimmock 1975, 1977; Lamb & Choppin 1978; Lamb *et al.* 1978). We have found a polypeptide (4) of molecular mass *ca.* 11000 in a wide variety of cell types infected with the WSN strain of influenza A virus. That this peptide is virus-specific was shown by strain-dependent differences in the mobility of the polypeptides of several different strains of influenza A virus in the same cell type. A polypeptide with a similar molecular mass has also been found in cells infected with influenza B virus. Extensive tryptic peptide mapping analyses have shown that the polypeptide of molecular mass *ca.* 11000 in WSN infected cells is distinct from all of the other eight virus-specific polypeptides (Lamb *et al.* 1978). The available evidence suggests that the mRNA for polypeptide 4 is not made by primary transcription, and that early protein synthesis is required

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

for its synthesis. Fractionation of infected cells showed that polypeptide 4 was predominantly cytoplasmic in location. A very small amount of this polypeptide has also been found in purified virions, and shown to be similar to polypeptide 4 in infected cells by peptide mapping. The polypeptide is resistant to proteolytic enzyme treatment of virions, suggesting a location within the viral membrane. Slight mobility differences between the polypeptides obtained from infected cells and virions have been observed, raising the possibility of secondary modification, but we could not show that polypeptide 4 was phosphorylated under conditions in which NP and NS were phosphorylated.

As we have shown that polypeptide 4 is a unique, ninth influenza virus polypeptide, and that a polyadenylated mRNA of *ca.* 300–400 nucleotides can be obtained from infected cells which codes for this polypeptide, and as this mRNA apparently hybridizes to one of the eight genome RNA segments, the question arises as to the origin of this specific mRNA. The data would suggest that one of the eight influenza virus RNA segments may code for two polypeptide products. The RNA species of influenza virus correspond in size to the polypeptides for which they code, with small exceptions in the RNAs coding for the P polypeptides (McGeoch *et al.* 1976; Desselberger & Palese 1978). These differences in the P-coding RNAs suggested that transcription of the mRNA for polypeptide 4 might originate from a second initiation site either in the same or a different reading frame on one of the P RNAs (Lamb *et al.* 1978). However, we have not been able to obtain hybridization of the mRNA to any of the three P RNA species. Therefore, the mRNA for the polypeptide 4 presumably initiates on one of the other RNA species. If the size estimates of the RNA species and the polypeptide products are correct, this hypothesis would also suggest that the translation of 4 is in a different reading frame from the other gene product.

Although the above scheme for the synthesis of polypeptide 4 in normal infections is attractive, we cannot totally exclude the possibility that the mRNA for polypeptide 4 is produced by the above mechanisms as a consequence of a virus–host cell interaction leading to defective replication. However, the observation that polypeptide 4 is unique and has a separate mRNA although there are only eight virus genes requires considerable further study. The possible biological function of this polypeptide merits special attention.

Note added in proof (14 *November* 1979). Using recombinants of strains PR8 and HK of defined gene composition and utilizing the electrophoretic mobility differences of NS_2 between these strains, we have shown that the gene for NS_2 reassorts with that of the non-structural polypeptide NS_1. Hybridization of genome segment 8 to the total mRNA specifically prevents the synthesis *in vitro* of NS_2 and NS_1. Thus, these results indicate that the influenza virus genome segment 8 is transcribed into two separate mRNAs that code for polypeptides NS_1 and NS_2 (Lamb, R. A. & Choppin, P. W. 1979 *Proc. natn. Acad. Sci. U.S.A.* **76**, 4908–4912).

We thank Mary-Louise Scully and Cathleen O'Connell for excellent technical assistance. This research was supported by Research Grants AI-05600 from the National Institute of Allergy and Infectious Diseases and PCM 76-09993 from the National Science Foundation. R.A.L. is an Irma T. Hirschl Career Scientist Awardee.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Lamb & Choppin)

Barry, R. D. & Mahy, B. W. J. 1979 The influenza virus genome and its replication. *Br. med. Bull.* **35**, 39–46.

Choppin, P. W., Lazarowitz, S. G. & Goldberg, A. R. 1975 Studies on proteolytic cleavage and glycosylation of the haemagglutinin of influenza A and B viruses. In *Negative strand viruses* (ed. B. W. J. Mahy & R. D. Barry), vol. 1, pp. 105–119. London: Academic Press.

Desselberger, U. & Palese, P. 1978 Molecular weights of RNA segments of influenza A and B viruses. *Virology* **88**, 394–399.

Etkind, P. R. & Krug, R. M. 1975 Purification of influenza viral complementary RNA: its genetic content and activity in wheat germ cell-free extracts. *J. Virol.* **16**, 1464–1475.

Follett, E. A. C., Pringle, C. R., Wunner, W. H. & Skehel, J. J. 1974 Virus replication in enucleate cells: vesicular stomatitis virus and influenza virus. *J. Virol.* **13**, 394–399.

Inglis, S. C., Carroll, A. R., Lamb, R. A. & Mahy, B. W. J. 1976 Polypeptides specified by the influenza virus genome. 1. Evidence for eight distinct gene products specified by fowl plague virus. *Virology* **74**, 489–503.

Lamb, R. A. & Choppin, P. W. 1976 Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. *Virology* **74**, 504–519.

Lamb, R. A. & Choppin, P. W. 1977*a* The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. *Virology* **81**, 382–397.

Lamb, R. A. & Choppin, P. W. 1977*b* Synthesis of influenza virus polypeptides in cells resistant to alpha-amanitin: evidence for the involvement of cellular RNA polymerase II in virus replication. *J. Virol.* **23**, 816–819.

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* (ed. B. W. J. Mahy & R. D. Barry), pp. 229–238. London: Academic Press.

Lamb, R. A., Etkind, P. R. & Choppin, P. W. 1978 Evidence for a ninth influenza viral polypeptide. *Virology* **91**, 60–78.

Lazarowitz, S. G., Compans, R. W. & Choppin, P. W. 1971 Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes. *Virology* **46**, 830–843.

McGeoch, D. J., Fellner, P. & Newton, C. 1976 The influenza virus genome consists of eight distinct RNA species. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3045–3049.

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.

Oxford, J. S. 1973 Polypeptide composition of influenza B viruses and enzymes associated with the purified virus particle. *J. Virol.* **12**, 827–835.

Peluso, R. W., Lamb, R. A. & Choppin, P. W. 1977 Polypeptide synthesis in simian virus 5-infected cells. *J. Virol.* **23**, 177–187.

Peluso, R. W., Lamb, R. A. & Choppin, P. W. 1978 Infection with paramyxoviruses stimulates synthesis of cellular polypeptides that are also stimulated in cells transformed by Rous sarcoma virus or deprived of glucose. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6120–6124.

Racaniello, V. R. & Palese, P. 1979 Influenza B virus genome: assignment of viral polypeptides to RNA segments. *J. Virol.* **29**, 361–373.

Scheid, A., Caliguiri, L. A., Compans, R. W. & Choppin, P. W. 1972 Isolation of paramyxovirus glycoproteins. Association of both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. *Virology* **50**, 640–652.

Skehel, J. J. 1972 Polypeptide synthesis in influenza virus-infected cells. *Virology* **49**, 23–36.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

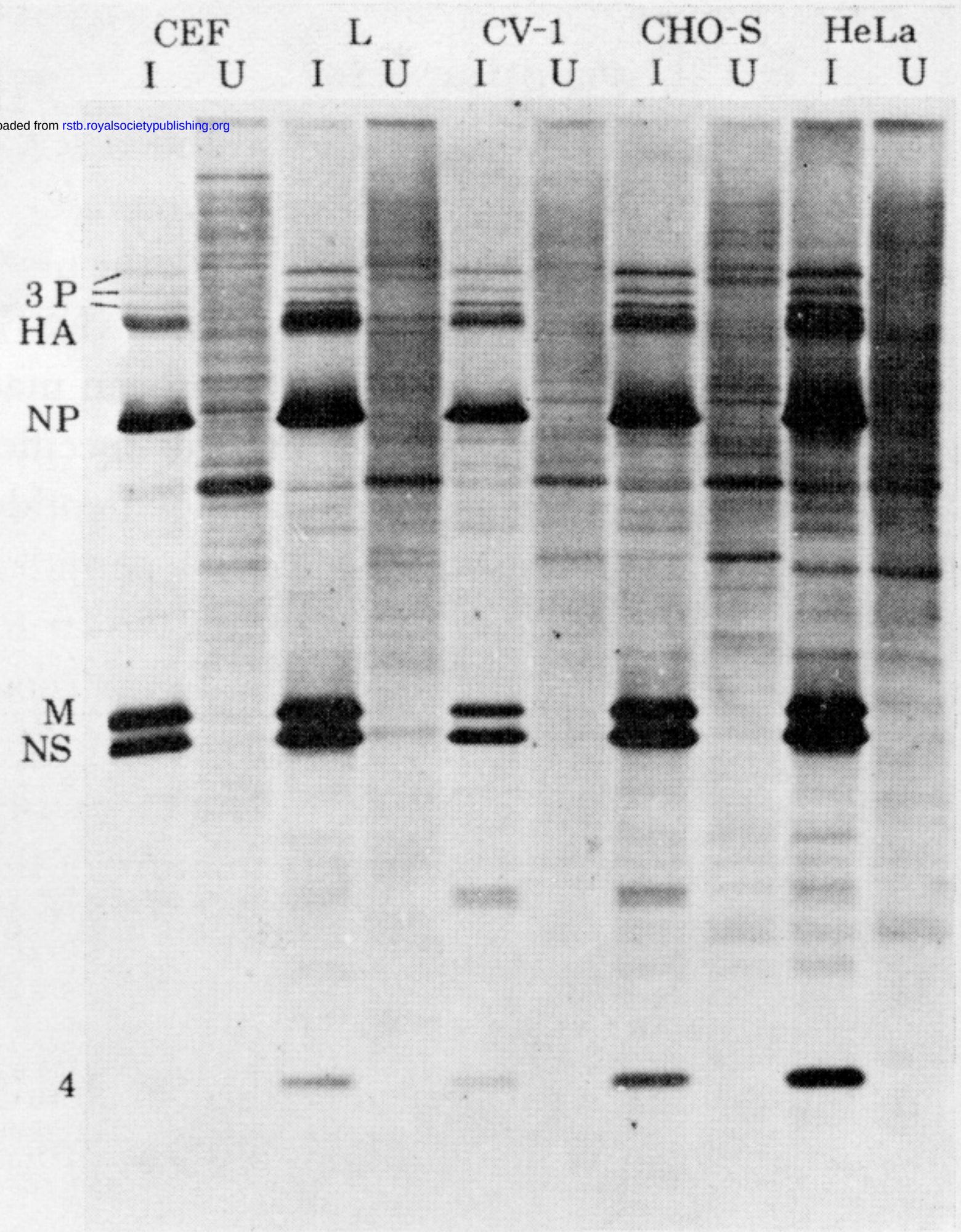

FIGURE 1. The synthesis of polypeptide 4 in WSN infected CEF, L, CV-1, CHO-S and HeLa cells. I, infected cells; U, uninfected cells (Lamb *et al.* 1978).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

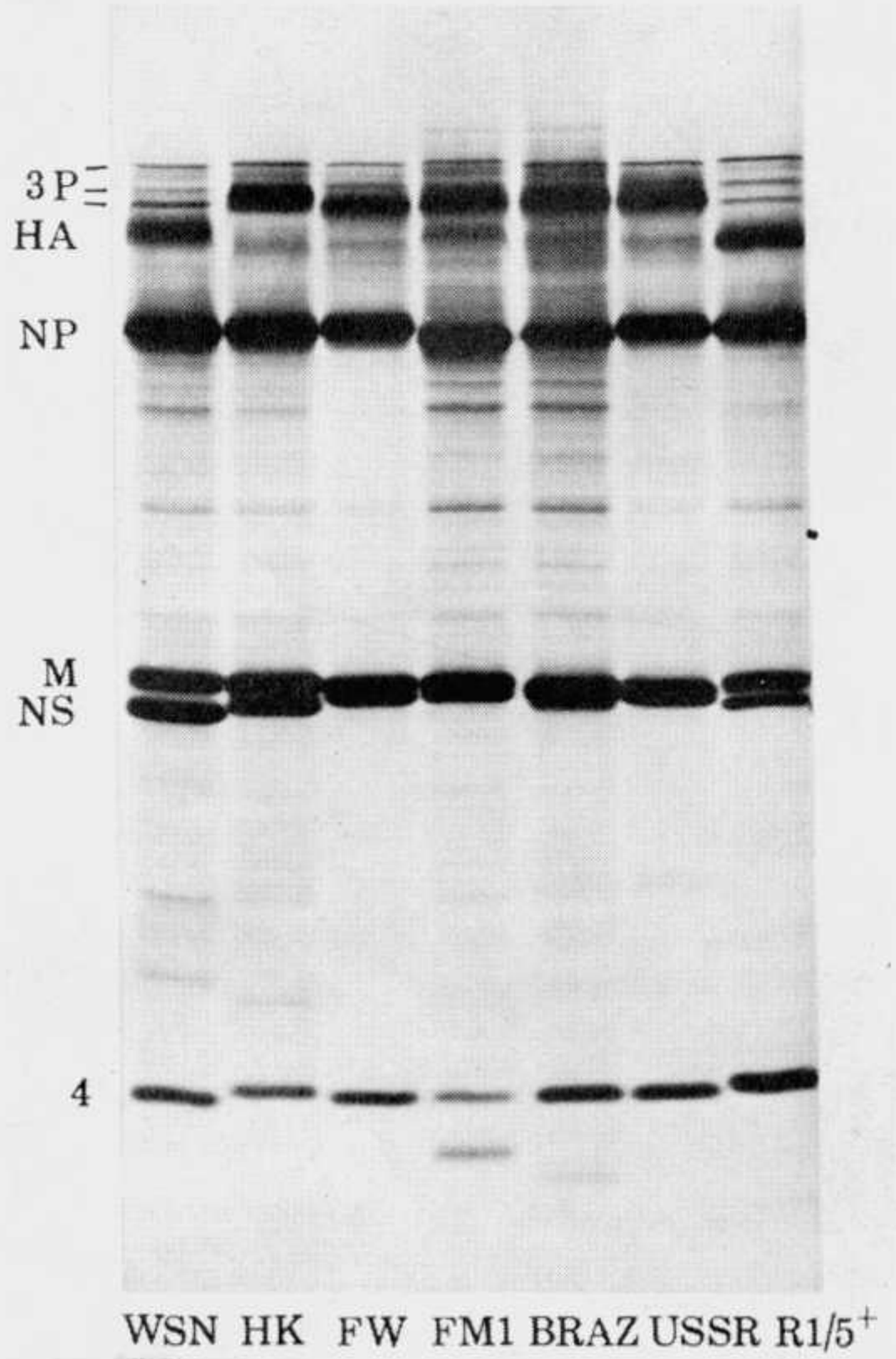

FIGURE 2. The synthesis of polypeptide 4 in HeLa cells infected with various strains of influenza A virus. At 6 h after infection, cells were labelled with [^{35}S]methionine (20 μCi/ml) for 30 min and subjected to electrophoresis on a 17.5% acrylamide, 4 M urea gel as described previously (Lamb *et al.* 1978). The strains used were, from left to right, WSN/33 (H0N1); Hong Kong/68 (H3N2); Fort Warren/50 (H1N1); FM1/47 (H1N1); Brazil/78 (H1N1); USSR/77 (H1N1); RI/5$^+$/57 (H2N2).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

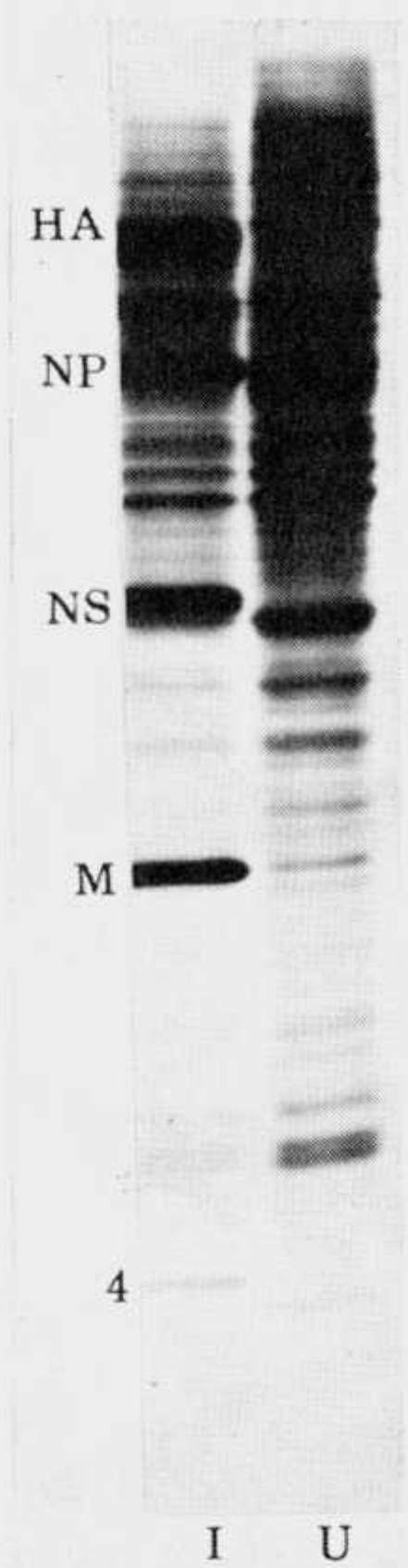

FIGURE 3. The synthesis of a polypeptide (4) of molecular mass *ca.* 11000 in influenza B/Lee infected HeLa cells. At 6 h after infection cells were labelled with [^{35}S]methionine (20 μCi/ml) for 30 min and subjected to electrophoresis on a 17.5% acrylamide, 4 M urea gel as described previously (Lamb *et al.* 1978).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

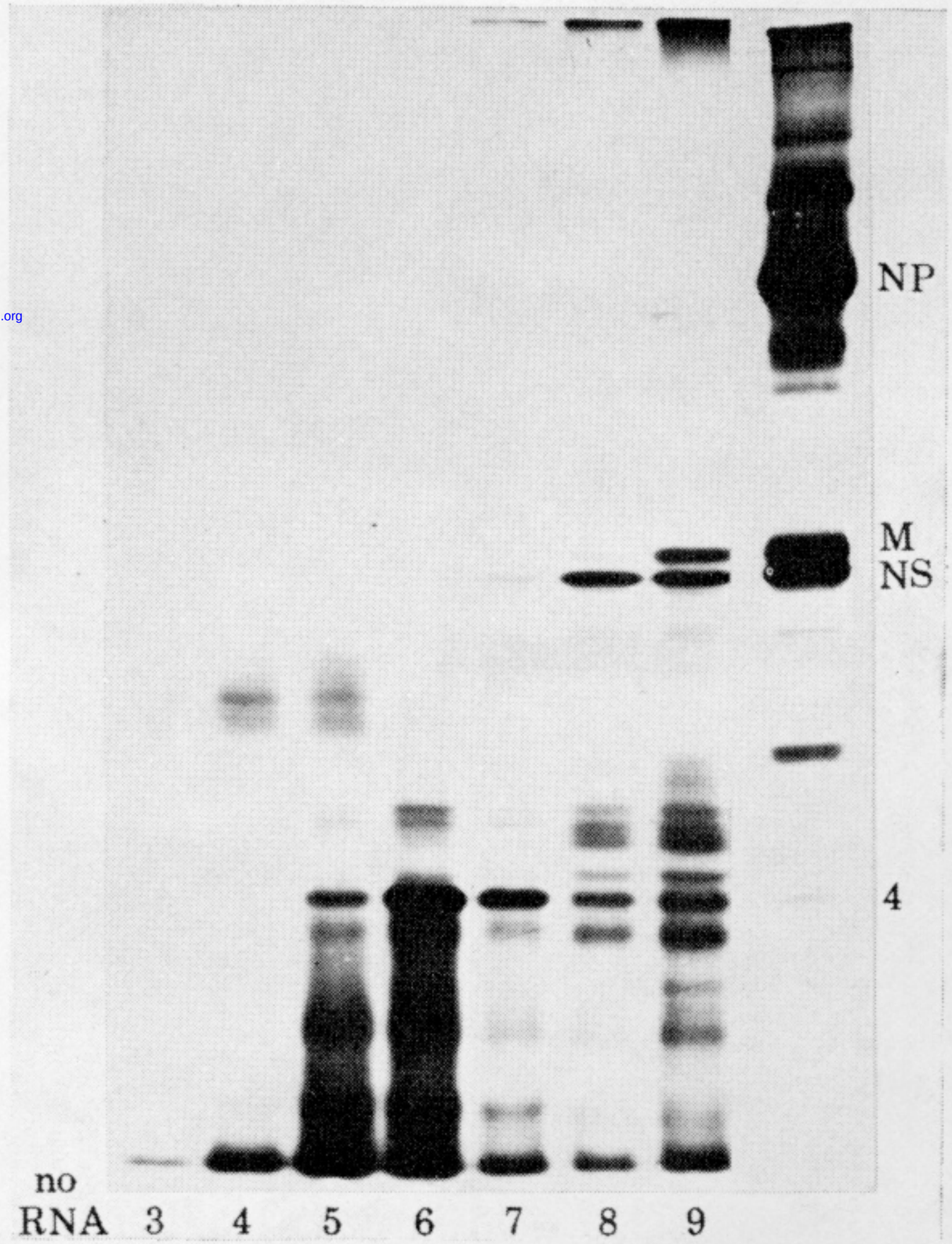

FIGURE 4. In-vitro translation of mRNAs from infected cells separated on sucrose density gradients. Poly(A)-containing RNA from the cytoplasm of WSN-infected CEF cells isolated 3 h after infection was dissolved in NETS buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 5 g/l SDS). The RNA was centrifuged for 20 h at 24000 rev/min at 22 °C in a 15–30 % (by mass) sucrose–NETS gradient in a Spinco SW27 rotor. Thirty fractions were collected and the RNA in each fraction translated in wheat germ extracts as described previously (Lamb *et al.* 1978). No RNA, no added RNA to wheat germ extract; lanes 3–9, translation of fractions 3–9. The far right lane contains WSN-infected CEF cell lysate to show viral polypeptides as markers.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

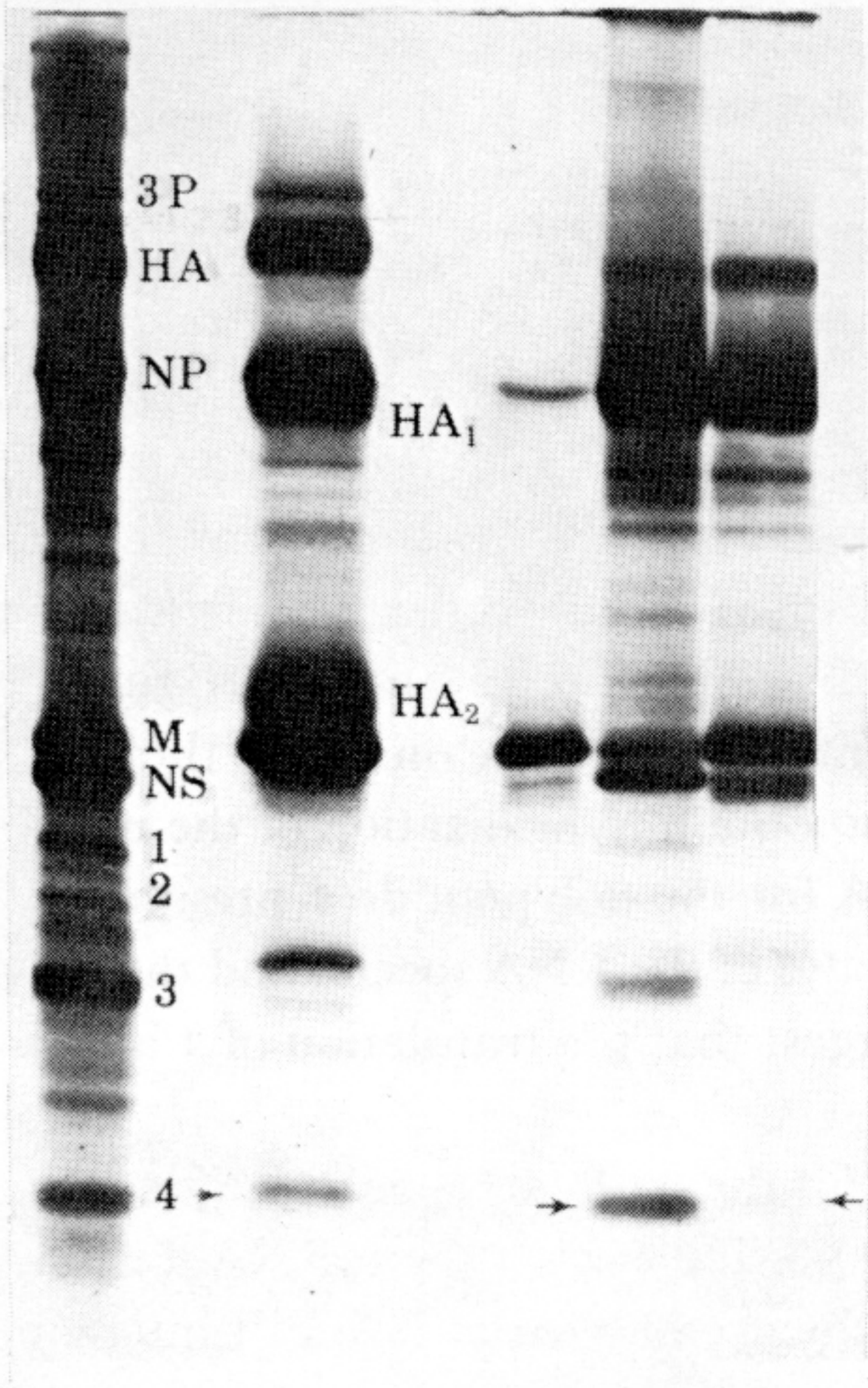

FIGURE 5. Immunoprecipitation of [^{35}S]methionine-labelled polypeptides from lysates of HeLa cells infected with the WSN strain of influenza A virus with antisera prepared against M protein, infected MDCK cells and virions, respectively. Lane 1, infected HeLa cell lysate, no antiserum; lane 2, purified marker virus; lane 3, cell lysates precipitated with antiserum to M; lane 4, cell lysate precipitated with antiserum to MDCK cells; lane 5, cell lysate precipitated with antiserum to purified virions (Lamb *et al.* 1978).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES