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Message Activity of Influenza Viral RNA

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The message activity of influenza virion RNA in the wheat germ cell-free protein-synthesizing system was investigated. RNA extracted from purified virions was found to direct the synthesis of a polypeptide that had the mobility of viral nucleocapsid protein on sodium dodecyl sulfate-polyacrylamide gels. Further characterization of the protein indicated it was not the nucleocapsid protein. No other polypeptides were detected. We conclude that influenza virion RNA is inactive as a template for the synthesis of virus-specific proteins.

The nature of the mRNA of influenza virus is a matter of some dispute. Most of the available evidence suggests that the majority of the message is complementary to viral RNA. The virus is known to contain transcriptase activity (4, 15) which makes RNA complementary to that in the virion (cRNA) (1). Pons (17, 18) has shown that most of the virus-specific RNA found on infected cell polyribosomes is complementary to viral RNA. Work by Etkind and Krug (6) supports this conclusion and shows that cRNA contains polyadenylate [poly(A)], whereas virion RNA (vRNA) does not. However, Nayak (14) has reported the presence of both cRNA and vRNA on infected cell polyribosomes.

Previous in vitro translation studies have been equally unclear. Kingsbury and Webster (9) found that RNA extracted from infected cells was active in a cell-free rabbit reticulocyte system. By using immune precipitation, he found two major products—one, a polypeptide that migrated with virus membrane protein (MP) in polyacrylamide gels; the other, a protein that migrated as no known viral protein. In his studies, viral RNA was found to be inactive. On the other hand, work by Siegert et al. (23) using a cell-free *Escherichia coli* system, indicated that vRNA directed the synthesis of a protein of the same molecular weight as viral nucleocapsid protein (NP). Furthermore, this product appeared to be antigenically related to viral NP in double diffusion analysis.

To attempt to resolve these inconsistencies and to test the possibility that vRNA may code for one (or more) proteins while cRNA codes for the remainder, we have carefully examined the template activity of vRNA in a cell-free protein synthesis system derived from wheat germ.

The NWS strain of influenza virus was grown in 10-day-old embryonated chicken eggs inoculated with ~2 × 10^4 PFU of stock virus. Eggs were incubated at 37 C and harvested 40 h postinfection. Purification included clarification of fluids by low-speed centrifugation, followed by high-speed centrifugation for 2 h at 19,000 rpm in a Spinco type 19 rotor to pellet virus. The virus pellet was resuspended in flu buffer [10 mM Tris-chloride (pH 7.6)-100 mM NaCl-1 mM EDTA], sonically treated twice for

TABLE 1. *Stimulation of labeled amino acid incorporation (counts per minute) by influenza vRNA in a wheat germ cell-free system*[a]

Expt	No RNA added	Influenza RNA	+RNA/ −RNA
[^{3}H]Leu			
1	451	15,661	35
2	413	12,532	30
[^{3}H]Leu, [^{3}H]Phe, [^{3}H]Tyr			
1	1,528	23,543	15
2	1,591	23,947	15

[a] Wheat germ extracts were prepared from Niblack or Bar Rav wheat germ using the procedure of Marcu and Dudock (12) scaled up five times. The complete reaction in a final volume of 25 μl contained: 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 2 mM dithiothreitol, 100 mM KCl, 2.75 mM $MgAc_2$, 1 mM ATP (neutralized with KOH), 0.2 mM GTP (neutralized), 8.0 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 5 μl of wheat germ extract, 1.25 μCi of each ^{3}H-labeled amino acid ([^{3}H]leucine, 58 Ci/mmol; [^{3}H]tyrosine, 49 Ci/mmol; [^{3}H]phenylalanine, 16.5 Ci/mmol) (Amersham), and 30 μM of each of the remaining amino acids. Viral RNA was present at 204 μg/ml. Incubation was for 2 h at 30 C and was terminated by the addition of 1 ml of 5% trichloroacetic acid plus 0.5% appropriate unlabeled amino acid(s). Reactions were heated for 15 min at 90 C and then filtered through Whatman GF/c filters, dried, and counted in a toluene-based scintillant.

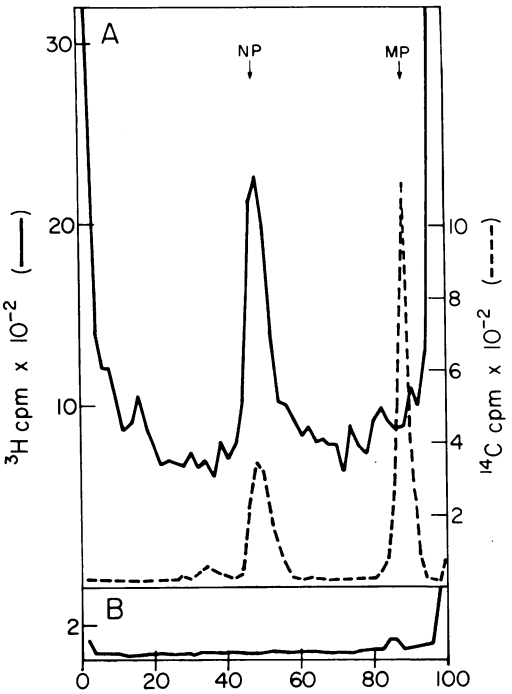

FIG. 1. *SDS-PAGE of cell-free products of (A) influenza vRNA-directed reaction and (B) endogenous reaction. Cell-free synthesis reactions incubated with [^{3}H]Tyr, [^{3}H]Leu, and [^{3}H]Phe were as described in the footnote to Table 1. Influenza vRNA was present at 230 μg/ml. Reactions were terminated by the addition of RNase A (20 μg/ml) and 10 mM EDTA (pH 7.5), followed by incubation for 10 min at 37 C. [^{14}C]Leu-, Tyr-, and Phe-labeled influenza virus were added, and samples were boiled for 2 to 3 min in 50 mM Tris-chloride (pH 6.8), 10% glycerol, 1% SDS, and 1% 2-mercaptoethanol. Slab gel electrophoresis used the discontinuous buffer system of Laemmli (10). A 10% acrylamide running gel and a 5% stacking gel were used. The gel was stained with Coomassie brilliant blue, destained, and dried. Each sample lane was cut out and sliced into 1-mm slices, and 50 μl of water and 10 ml of an NCS-containing toluene-based scintillant were added. After heating overnight at 37 C to solubilize the counts, gels were counted. ^{14}C-labeled virus was prepared by infecting MDCK cells at a multiplicity of infection of 1 in Dulbecco modified Eagle medium (DME-HEPES) plus 2% calf serum. At 4 h postinfection, the medium was removed and labeling medium (no Leu, Tyr, Phe) plus 2% dialyzed calf serum was added. [^{14}C]Leu (240 mCi/mmol) (ICN), [^{14}C]Tyr (360 mCi/mmol) (ICN), and [^{14}C]Phe (360 mCi/mmol) (ICN) were added at 0.5 μCi/ml. At 6 h postinfection, cold Leu, Tyr, and Phe were added to 0.1 concentration present in DME-HEPES. Virus was harvested 40 h postinfection and purified as described.*

5 s at setting 4 on a Branson sonifier W185 with a microtip, and then incubated for 20 min at 37 C with 20 μg each of neuraminidase and RNase A per ml. Virus was equilibrium banded in 5 to 30% (wt/wt) gradients of sodium-potasssium-tartrate in flu buffer, and the virus band was collected, diluted with flu buffer, and pelleted by ultracentrifugation at 28,000 rpm for 1 h in a type 30 rotor. Purified virus was resuspended in the buffer, and RNA was extracted by a modification of the procedure of Duesberg and Robinson (5). Phenol equilibrated with flu buffer was used for the extractions. Phenol was removed from the aqueous phase by extraction with an equal volume of ether. Residual ether was removed by blowing a stream of air across the aqueous phase. RNA was preciptated as described. Precipitated RNA was dissolved in 0.05 M sodium acetate (pH 5.0) plus 0.15 M NaCl and reprecipitated. Reprecipitated RNA was dissolved in sterile water.

Viral RNA was found to stimulate the incorporation of labeled amino acids into trichloroacetic acid-precipitable material (Table 1). Stimulation of incorporation was maximal at a mag-

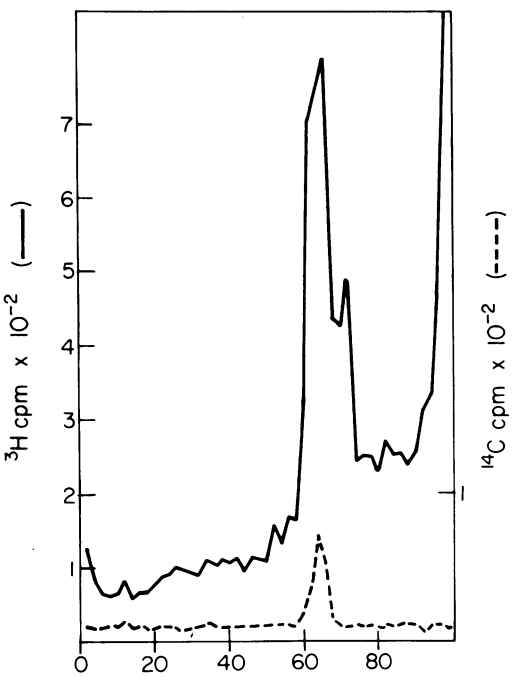

FIG. 2. *SDS-PAGE of cell-free products directed by self-annealed influenza RNA. Annealing was a modification of the method of Robinson et al. (20). A 17-μg amount of RNA in 30 μl of annealing buffer (0.02 M Tris-chloride [pH 7.4]-0.2 M KCl) was sealed in glass tubes and annealed by slow cooling from 85 C to room temperature. An aliquot was used to test message activity in the cell-free wheat germ system as described. Products of the reaction were analyzed as described in the legend to Fig. 1. ^{14}C-labeled viral N protein, prepared by elution from preparative gels of ^{14}C-labeled virus, was run in the same lane as an internal marker.*

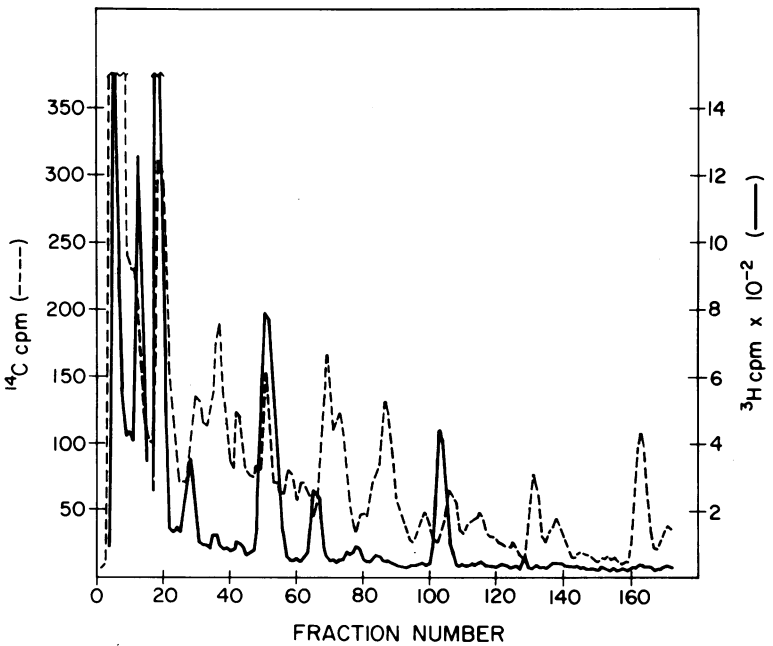

FIG. 3. *Aminex A-5 chromatography of a tryptic digest of ^{3}H-labeled in vitro synthesized N-like protein and ^{14}C-labeled viral N protein. [^{3}H]Leu-, Tyr-, and Phe-labeled cell-free reactions directed by vRNA and [^{14}C]-Leu, Tyr-, and Phe-labeled influenza virus were run together on SDS-PAGE. The gel was sliced into 2-mm slices, and 0.3 ml of elution buffer (10 mM Tris-chloride [pH 8], 50 mM Nacl, 1 mM EDTA, 0.1% SDS, and 0.1% 2-ME) was added to each slice. Elution was accomplished by shaking overnight at 37 C. An aliquot was counted and appropriate fractions were pooled. Bovine serum albumin was added at 0.5 mg/ml, and the protein was precipitated by adding two volumes of 95% EtOH. The precipitate was spun down and dissolved in 0.1 M NH_4HCO_3 (pH 8.0) and digested for 24 h at room temperature with TPCK-trypsin (Worthington). Trypsin was added twice over the digestion period, such that the total amount of trypsin added was 5% of the total protein. The reaction was terminated by lyophilization. The residue was dissolved in 2% formic acid and applied to an Aminex A-5 (Bio-Rad) column (0.9 by 20 cm) equilibrated with 0.2 M pyridinium acetate (pH 3.0). Column chromatography was carried out at 50 C under a pressure head of 50 lb/in^2. Elution was effected with an exponential gradient, 350 ml of 0.2 M pyridinium acetate (pH 3.0) and 160 ml of 2 M pyridinium acetate (pH 5.0). Fractions (3 ml) were collected and processed by evaporating each fraction to dryness, redissolving in 1 ml of water, and counting in 10 ml of a Triton-X toluene-based liquid scintillant.*

nesium concentration of 2.75 mM and at a potassium concentration of 100 mM. Analysis of the in vitro products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated the presence of a single polypeptide that had the same electrophoretic mobility as viral NP (Fig. 1A). No discrete polypeptides were detected in the analysis of the endogenous reaction (Fig. 1B). Each of eight different viral RNA preparations tested was found to direct the synthesis of this N-like polypeptide.

Although it has been previously shown that influenza virions contain only vRNA (16, 21), annealing experiments were performed and the effect on message activity in the wheat germ system was determined. No stimulation in message activity was found upon boiling and quick cooling of the RNA, as would be expected if cRNA were the mRNA and complexed with vRNA. Furthermore, self-annealing of the RNA did not abolish the ability of the RNA to direct the synthesis of the N-like protein (Fig. 2), as would be expected if the mRNA were a trace contamination of uncomplexed cRNA.

To determine whether the product synthesized in vitro was authentic viral N protein, a tryptic digestion was performed, and the peptides were compared by column chromatography on Aminex A-5. As shown in Fig. 3, the tryptic peptides of the in vitro product and authentic viral N protein were found to be almost completely different, suggesting nonidentity of the two proteins.

To rule out the possibility that the wheat germ extract was not capable of accurate trans-

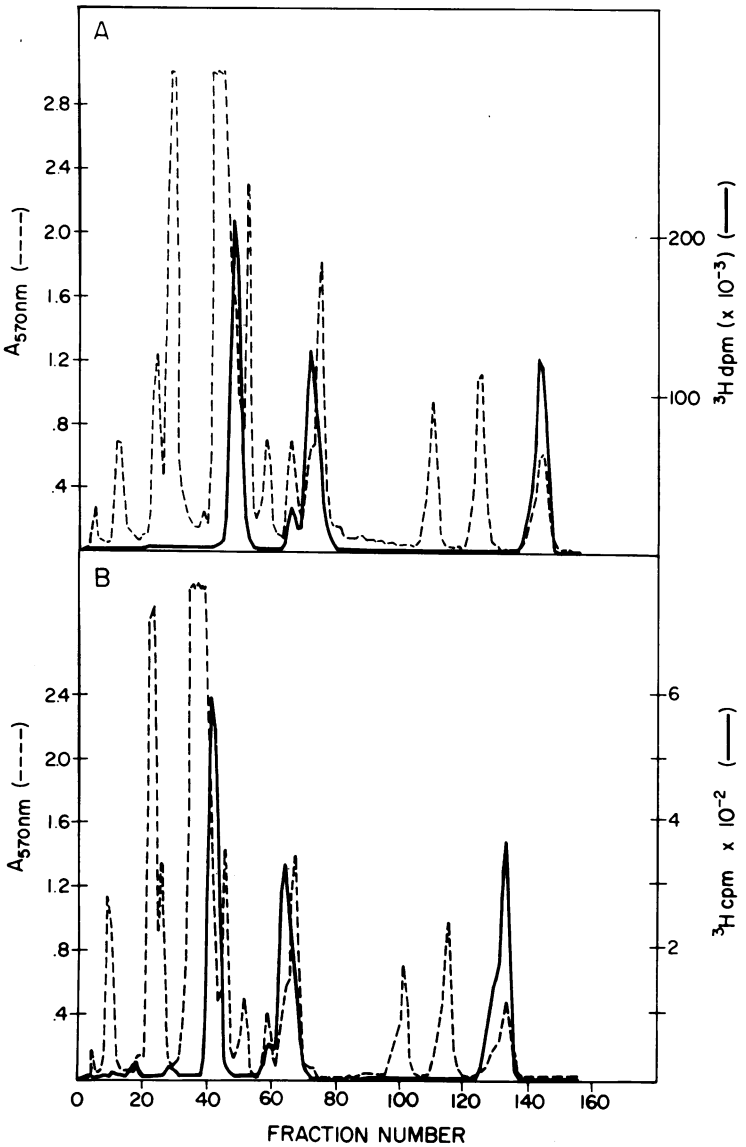

FIG. 4. *Aminex A-5 chromatography of tryptic digests of the α chain of hemoglobin made in vitro in an (A) rabbit reticulocyte and (B) wheat germ cell-free system. (A) Reticulocyte lysates were prepared by the method of Gilbert and Anderson (8). Endogenous synthesis was carried out as described by Villa-Komaroff et al. (24) using [^{3}H]tyrosine as a label. (B) Rabbit reticulocyte polysomes were prepared as described by Lebleu (11). Total RNA was extracted from polysomes by the SDS-phenol method of Brawerman et al. (3). Globin mRNA was isolated by chromatography of total RNA on Sigma cell type 50 cellulose as described by Schultz et al. (22). Globin mRNA maximally stimulated the incorporation of [^{3}H]tyrosine into trichloroacetic acid-precipitable material in the wheat germ cell-free system at [Mg^{2+}] of 3.0 mM and [K^+] of 60 mM. In both the above cases, 400 mg of crude rabbit hemoglobin was added as carrier to the in vitro reaction. α chain was isolated as described (25), and trypsin digestion was carried out as described in the legend to Fig. 3. Analysis of tryptic peptides was carried out by column chromatography on Aminex A-5 under the following conditions. The pressure on the column was 100 lb/in^2. Elution was accomplished by means of a linear gradient: 350 ml of 0.2 M pyridinium acetate (pH 3.0) and 350 ml of 2 M pyridinium acetate (pH 5.0). Fractions (4 ml) were collected, and a 0.2-ml aliquot was taken for ninhydrin assay; ninhydrin color was read at 570 nm. The remainder of each fraction was processed as described in the legend to Fig. 3.*

lation of an mRNA, globin mRNA was isolated and translated. The tyrosyl-containing peptides of the α-globin chain synthesized by the globin mRNA-directed wheat germ system and the rabbit reticulocyte system were compared (Fig. 4). The α-globin chain made in each system contained labeled tyrosine in the same three tryptic peptides. These peptides are known from sequence studies to contain all the tyrosine present in the molecule (13). These experiments demonstrate that our wheat germ cell-free system is capable of very accurate translation of mRNA. Occasional premature termination of polypeptide chains is known to occur in the wheat germ cell-free system (2, 19) but is believed not to pertain here due to the absence of any significant amount of a larger protein.

From these experiments we conclude that influenza viral RNA is inactive as a template for the synthesis of any of the known influenza proteins. Our work supports the conclusion of Etkind and Krug (7), that all of the message activity for this virus resides in cRNA. Whether the N-like polypeptide synthesized in our system (and perhaps that of Siegert et al. [23]) is the product of a host cell message selectively incorporated into the virion or results from efficient mistranslation of the vRNA is not known. In either case, our results emphasize the desirability of definitive characterization of proteins produced in vitro from putative mRNA molecules.

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LITERATURE CITED

1. **Bishop, D. H. L., P. Roy, W. J. Bean, Jr., and R. W. Simpson.** 1972. Transcription of the influenza RNA genome by a virion polymerase. J. Virol. 10:689–697.
2. **Both, G. W., S. Lavi, and A. J. Shatkin.** 1975. Synthesis of all the gene products of the reovirus genome *in vivo* and *in vitro*. Cell 4:173–180.
3. **Brawerman, G., J. Mendecki, and S. V. Lee.** 1972. A procedure for the isolation of mammalian messenger ribonucleic acid. Biochemistry 11:637–641.
4. **Chow, N. L., and R. W. Simpson.** 1971. RNA-dependent RNA polymerase activity associated with virions and subviral particles of myxoviruses. Proc. Natl. Acad. Sci. U.S.A. 68:752–756.
5. **Duesberg, P. H., and N. S. Robinson.** 1965. Isolation of nucleic acid of Newcastle Disease virus. Proc. Natl. Acad. Sci. U.S.A. 54:794–800.
6. **Etkind, P. R., and R. M. Krug.** 1974. Influenza viral messenger RNA. Virology 62:38–45.
7. **Etkind, P. R., and R. M. Krug.** 1975. Purification of influenza viral complementary RNA: its genetic content and activity in wheat germ cell-free extracts. J. Virol. 16:1464–1475.
8. **Gilbert, J. M., and W. F. Anderson.** 1970. Cell-free hemoglobin synthesis. II. Characteristics of the transfer ribonucleic acid-dependent assay system. J. Biol. Chem. 245:2342–2349.
9. **Kingsbury, D. W., and R. G. Webster.** 1973. Cell-free translation of influenza virus messenger RNA. Virology 56:654–657.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
11. **Lebleu, B.** 1974. Isolation of rabbit reticulocyte 9S mRNA, p. 613–621. *In* K. Moldave and L. Grossman (ed.), Methods in enzymology, vol. 30. Academic Press Inc., New York.
12. **Marcu, K., and B. Dudock.** 1974. Characterization of a highly efficient protein synthesizing system derived from commercial wheat germ. Nucleic Acid Res. 1:1385–1397.
13. **Mushinski, J. F., A. Galizzi, and G. von Ehrenstein.** 1970. Cell-free transfer of leucine by transfer ribonucleic acid from mouse liver and plasma cell tumors into rabbit hemoglobin. Biochemistry 9:489–495.
14. **Nayak, D. P.** 1970. The replication of influenza virus RNA, p. 371–391. *In* R. D. Barry and B. W. J. Mahy (ed.), Biology of large RNA viruses. Academic Press Inc., New York.
15. **Penhoet, E. E., H. Miller, M. Doyle, and S. Blatti.** 1971. RNA-dependent RNA polymerase activity in influenza virions. Proc. Natl. Acad. Sci. U.S.A. 68:1369–1371.
16. **Pons, M. W.** 1971. Isolation of influenza virus ribonucleoprotein from infected cells. Demonstration of the presence of negative stranded RNA in viral RNP. Virology 46:149–160.
17. **Pons, M. W.** 1972. Studies on the replication of influenza virus RNA. Virology 47:823–832.
18. **Pons, M. W.** 1975. Influenza virus messenger ribonucleoprotein. Virology 67:209–218.
19. **Prives, C. L., H. Aviv, B. M. Paterson, B. E. Roberts, S. Rozenblatt, M. Revel, and E. Winocour.** 1974. Cell-free translation of messenger RNA of simian virus 40: synthesis of the major capsid protein. Proc. Natl. Acad. Sci. U.S.A. 71:302–306.
20. **Robinson, W. S., W. T. Hsu, C. F. Fox, and S. B. Weiss.** 1964. Enzymatic synthesis of ribonucleic acid. IV. The deoxyribonucleic acid-directed synthesis of complementary cytoplasmic ribonucleic acid components. J. Biol. Chem. 239:2944–2951.
21. **Scholtissek, C., and H. Becht.** 1971. Binding of ribonucleic acids to the RNP antigen protein of influenza viruses. J. Gen. Virol. 10:11–16.
22. **Schultz, G., M. Beato, and P. Feigelson.** 1972. Isolation of eukaryotic messenger RNA on cellulose and its translation in vitro. Biochem. Biophys. Res. Commun. 49:680–689.
23. **Siegert, W., G. Bauer, and P. H. Hofschneider.** 1973. Direct evidence for messenger activity of influenza virion RNA. Proc. Natl. Acad. Sci. U.S.A. 70:2960–2963.
24. **Villa-Komaroff, L., M. McDowell, D. Baltimore, and H. Lodish.** 1974. Translation of reovirus mRNA, poliovirus RNA and bacteriophage QB RNA in cell-free extracts of mammalian cells, p. 709–723. *In* K. Moldave and L. Grossman (ed.), Methods in enzymology, vol. 30. Academic Press Inc., New York.
25. **Weisblum, B., F. Gonano, G. von Ehrenstein, and S. Benzer.** 1965. A demonstration of coding degeneracy for leucine in the synthesis of protein. Proc. Natl. Acad. Sci. U.S.A. 53:328–334.