Edited by Heinz Fraenkel-Conrat and Robert R. Wagner

Comprehensive Virology

10

Regulation and Genetics

Viral Gene Expression and Integration

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Foreword

The time seems ripe for a critical compendium of that segment of the biological universe we call viruses. Virology, as a science, having passed only recently through its descriptive phase of naming and numbering, has probably reached that stage at which relatively few new—truly new—viruses will be discovered. Triggered by the intellectual probes and techniques of molecular biology, genetics, biochemical cytology, and high-resolution microscopy and spectroscopy, the field has experienced a genuine information explosion.

Few serious attempts have been made to chronicle these events. This comprehensive series, which will comprise some 6000 pages in a total of about 22 volumes, represents a commitment by a large group of active investigators to analyze, digest, and expostulate on the great mass of data relating to viruses, much of which is now amorphous and disjointed, and scattered throughout a wide literature. In this way, we hope to place the entire field in perspective, and to develop an invaluable reference and sourcebook for researchers and students at all levels.

This series is designed as a continuum that can be entered anywhere, but which also provides a logical progression of developing facts and integrated concepts.

Volume 1 contains an alphabetical catalogue of almost all viruses of vertebrates, insects, plants, and protists, describing them in general terms. Volumes 2–4 deal primarily, but not exclusively, with the processes of infection and reproduction of the major groups of viruses in their hosts. Volume 2 deals with the simple RNA viruses of bacteria, plants, and animals; the togaviruses (formerly called arboviruses), which share with these only the feature that the virion's RNA is able to act as messenger RNA in the host cell; and the reoviruses of animals and plants, which all share several structurally singular features, the most important being the double-strandedness of their multiple RNA molecules.

Volume 3 addresses itself to the reproduction of all DNA-containing viruses of vertebrates, encompassing the smallest and the largest viruses known. The reproduction of the larger and more complex RNA viruses is the subject matter of Volume 4. These viruses share the property of being enclosed in lipoprotein membranes, as do the togaviruses included in Volume 2. They share as a group, along with the reoviruses, the presence of polymerase enzymes in their virions to satisfy the need for their RNA to become transcribed before it can serve messenger functions.

Volumes 5 and 6 represent the first in a series that focuses primarily on the structure and assembly of virus particles. Volume 5 is devoted to general structural principles involving the relationship and specificity of interaction of viral capsid proteins and their nucleic acids, or host nucleic acids. It deals primarily with helical and the simpler isometric viruses, as well as with the relationship of nucleic acid to protein shell in the T-even phages. Volume 6 is concerned with the structure of the picornaviruses, and with the reconstitution of plant and bacterial RNA viruses.

Volumes 7 and 8 deal with the DNA bacteriophages. Volume 7 concludes the series of volumes on the reproduction of viruses (Volumes 2–4 and Volume 7) and deals particularly with the single- and double-stranded virulent bacteriophages.

Volumes 8, the first of the series on regulation and genetics of viruses, covers the biological properties of the lysogenic and defective phages, the phage-satellite system P 2–P 4, and in-depth discussion of the regulatory principles governing the development of selected lytic phages.

Volume 9 provides a truly comprehensive analysis of the genetics of all animal viruses that have been extensively studied to date. Described in ten detailed chapters are genotypes and phenotypic expression of conditional, host range, and deletion mutants of three major classes of animal DNA viruses followed by seven genera of RNA viruses. Principles and methodology are presented and compared to provide insight into mechanisms of mutagenesis, selection of mutants, complementation analysis, and gene mapping with restriction endonucleases and other methods. Whenever appropriate, the genetic properties of viruses are related to nucleic acid structure and function as well as recombination, integration of viral with host genome, malignant transformation, and alteration of host cell functions.

The present volume deals with transcriptional and translational regulation of viral gene expression, defective virions, and integration of tumor virus genomes into host cell chromosomes. Later volumes will be

concerned with regulation of plant virus development, covirus systems, satellitism, and viroids. Two or three additional volumes will be devoted largely to structural aspects and the assembly of bacteriophages and animal viruses, as well as to special groups of newer viruses.

The complete series will endeavor to encompass all aspects of the molecular biology and the behavior of viruses. We hope to keep this series up to date at all times by prompt and rapid publication of all contributions, and by encouraging the authors to update their chapters by additions or corrections whenever a volume is reprinted.

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CHAPTER 1

Translation of Animal Virus mRNAs *in Vitro*

A. J. Shatkin, A. K. Banerjee, and G. W. Both
Roche Institute of Molecular Biology
Nutley, New Jersey 07110

1. INTRODUCTION

Studies of the translation of bacteriophage RNAs in cell-free systems have contributed much to our understanding of many important aspects of protein synthesis, e.g., the elucidation of RNA virus gene order and the mechanism of suppression of nonsense mutations. Cell-free systems have also been useful for studying the regulation of cellular polypeptide formation, notably the role of cAMP and its binding protein in the expression of the *gal* operon. Recently, eukaryotic cellular and viral mRNAs that contain 3′-terminal poly(A) have been purified by selective binding to oligo(dT)-cellulose or poly(U)-sepharose. In addition, many animal virus mRNAs can be prepared in large quantities *in vitro* by taking advantage of the respective virion-associated transcriptases, and several heterologous cell-free systems synthesize authentic viral and cellular proteins in response to these purified mRNAs. The potential of *in vitro* systems for studying eukaryotic gene expression is likely to continue to attract the interest and attention of increasing numbers of investigators. It therefore seems appropriate to consider some of the basic characteristics of the cell-free systems that are available as of June 1975, when this chapter was written, to summarize the results of current studies, and to discuss how future work on

the *in vitro* translation of animal virus mRNAs may increase our knowledge of the biochemistry of animal virus multiplication and eukaryotic cell growth.

2. PREPARATION AND COMPARATIVE PROPERTIES OF *IN VITRO* PROTEIN-SYNTHESIZING SYSTEMS

The basic mechanisms of prokaryotic protein synthesis are now reasonably well understood (Lucas-Lenard and Lipmann, 1971), and in many ways protein synthesis in eukaryotes is similar (Haselkorn and Rothman-Denes, 1973). With a general understanding of this process and with hindsight gained from work with prokaryotes, it has been possible to prepare *in vitro* protein-synthesizing systems from a variety of eukaryotic cells and organisms. As discussed in this chapter, many viral mRNAs have recently been isolated and can be faithfully translated in these systems. As such, *in vitro* translation is a particularly useful method for identifying an unknown mRNA on the basis of the protein for which it codes. Moreover, the primary translation product of a purified viral mRNA can often be identified in a cell-free system whereas it may be lost by rapid proteolytic cleavage *in vivo*. This information is helpful in elucidating the events which take place in virus-infected cells.

In this section, the general properties of cell-free protein-synthesizing systems derived from frog eggs and oocytes, tissue culture and ascites cells, reticulocytes, and wheat germ will be compared with emphasis on the basic methods for preparing active extracts, their efficiencies of translation, and the relative advantages and disadvantages of each system. In this chapter, an *in vitro* protein-synthesizing system (*in vitro* or cell-free system for short) is defined as one in which amino acid incorporation into authentic polypeptides is directed by exogenous messenger RNA. Although eggs and oocytes are not, strictly speaking, *in vitro* systems and certainly not cell free, they have been used for translating viral mRNAs and are therefore included in this discussion.

2.1. Frog Eggs and Oocytes

The preparation of frog oocytes for studies on protein synthesis was first described with the South African clawed toad, *Xenopus laevis*, by Gurdon (1968), but oocytes have also been obtained from *Pleurodeles waltlii* (Brachet *et al.*, 1973) and the Queensland cane toad,

Bufo marinus (May and Glenn, 1974). Oocytes from the former are larger than those of *Xenopus* and are better able to survive microinjection (Brachet *et al.*, 1973). The *Bufo* species is widely distributed and, unlike *Xenopus*, is found on all continents of the world (May and Glenn, 1974). Active oocytes from ovarian tissue and unfertilized eggs (Gurdon, 1967) are obtained from sacrificed female frogs which had been induced to ovulate by hormonal injection between 2 and 4 weeks previously (Gurdon *et al.*, 1971). A radioactive amino acid or mRNA is introduced by injection with a micropipette into actively growing oocytes (May and Glenn, 1974; Gurdon *et al.*, 1971). The oocytes are then incubated in culture medium at 19°C, where they remain synthetically active for up to 3 days (Gurdon, 1968). Radioactive amino acids can also be introduced into the cells by addition to the culture medium. The choice of labeling procedure is determined mainly by (1) the rate at which the label in the culture medium penetrates the cells, (2) the rate at which injected label leaks out, and (3) the duration of the labeling period. Radioactive amino acid leaks out of injected oocytes more quickly than from injected eggs. However, the label (at least for [^{3}H]histidine) penetrates oocytes much more quickly than eggs. In general, when short labeling periods (up to 1 h) or unfertilized eggs are used, the highest amount of labeled protein is synthesized when the radioactive amino acid is injected. When oocytes are used, especially for labeling periods of more than 2 h, it is best to introduce label by incubation.

2.2. Reticulocytes

Circulating reticulocytes are collected by bleeding rabbits made anemic with daily subcutaneous injections of acetylphenylhydrazine (Adamson *et al.*, 1968; Housman *et al.*, 1970; Villa-Komaroff *et al.*, 1974*b*; Gilbert and Anderson, 1970). The blood is filtered through cheesecloth into chilled saline (Villa-Komaroff *et al.*, 1974*b*), and the cells are washed, packed by centrifugation, and lysed at 0°C by the addition of an equal volume of water (Adamson *et al.*, 1968; Housman *et al.*, 1970; Villa-Komaroff *et al.*, 1974*b*) or hypotonic buffer (Gilbert and Anderson, 1970; Schreier and Staehelin, 1973). After 60 s, the lysate is centrifuged at 30,000*g* for 15 min (Villa-Komaroff *et al.*, 1974*b*; Schreier and Staehelin, 1973) and the supernatant is frozen in aliquots at −80°C, at which temperature activity remains stable for several months.

The reticulocyte protein-synthesizing system has been extensively fractionated, and many of the factors involved in protein synthesis have been purified (see Vol. 30 of *Methods in Enzymology*, Academic Press, New York). One of the simplest fractionated systems derived from reticulocyte lysates and used to translate eukaryotic viral mRNA consists of a high-speed supernatant fraction (S100) derived by centrifugation of the reticulocyte lysate, ribosomes washed with 0.5 M KCl, and the ribosomal wash fraction (Cancedda and Schlesinger, 1974; Woodward *et al.*, 1974). This system preferentially translates exogenous viral mRNA (Cancedda and Schlesinger, 1974) and is more active than most of the more highly fractionated systems (Woodward *et al.*, 1974).

The most efficient mammalian cell-free protein-synthesizing system, described by Schreier and Staehelin (1973), was originally developed for the *in vitro* translation of exogenous rabbit globin mRNA. It is prepared by a procedure which maintains the structural and functional integrity of the ribosomes. The basic system consists of purified ribosomal subunits from mouse liver, rabbit reticulocytes, or guinea pig brain, partially purified initiation factors from rabbit reticulocytes, and elongation factors, termination factors, aminoacyl tRNA synthetases, and tRNA from rat liver in the form of pH 5 enzymes (Schreier and Staehelin, 1973). The system has been adapted for the translation of adenovirus-2-specific mRNA by the preparation of ribosomal subunits and the pH 5 enzyme fraction from ascites cells (Anderson *et al.*, 1974).

2.3. Ascites and Tissue Culture Cells

2.3.1. Propagation of Ascites Cells

Ascites tumor cells can be propagated in various strains of mice by intraperitoneal injection of 0.1–0.2 ml of ascitic fluid containing 5–10 $\times$ 10^7 cells/ml. Bright yellow fluid, which is probably contaminated with bacteria, or very bloody fluid should not be used for passaging (Mathews and Korner, 1970; Aviv *et al.*, 1971; Villa-Komaroff *et al.*, 1974*b*). The cells are harvested and propagated every 7–10 days (Martin *et al.*, 1961; Mathews and Korner, 1970; Aviv *et al.*, 1971; Jacobs-Lorena and Baglioni, 1972; McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*; Samuel and Joklik, 1974). One mouse provides 2–10 ml of fluid containing about 10^8 cells/ml (Martin *et al.*, 1961; Villa-Komaroff *et al.*, 1974*b*), and stocks can be frozen for recourse, should the cells in passage become unsuitable for use due to bloody or

clotted tumors (Mathews and Korner, 1970; Aviv *et al.*, 1971). Tumor cells can be passaged for up to 30–40 generations by this procedure without any obvious change in the relevant properties of the tumor (Mathews and Korner, 1970). In addition, ascites cells maintained in tissue culture in Eagle's medium retain the ability to cause tumors in animals (Van Venrooij *et al.*, 1970).

2.3.2. Growth of Tissue Culture Cells

Cell-free extracts used for translation of animal virus mRNAs have been prepared from HeLa cells (McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*; Eggen and Shatkin, 1972), Chinese hamster ovary (CHO) cells (McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*), mouse L-cell fibroblasts (McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*; Eggen and Shatkin, 1972; Friedman *et al.*, 1972*a*; Graziadei and Lengyel, 1972), and MOPC 460 tumor cells (Lawrence and Thach, 1974). Most of the cell lines can be grown in Eagle's minimum essential medium supplemented with 7–10% calf or bovine serum (McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*; Eggen and Shatkin, 1972; Friedman *et al.*, 1972*a*; Graziadei and Lengyel, 1972; Samuel and Joklik, 1974), but MOPC 460 tumor cells are grown in Liebowitz L15 medium (Lawrence and Thach, 1974). CHO cells should be further supplemented with nonessential amino acids (McDowell *et al.*, 1972). For the preparation of cell extracts, cultures are generally grown to densities of $2–10 \times 10^5$ cells/ml for L, HeLa, and CHO cells (McDowell *et al.*, 1972; Villa-Komaroff *et al.*, 1974*b*; Friedman *et al.*, 1972*a*; Graziadei and Lengyel, 1972) and $4–5 \times 10^4$ cells/ml for MOPC 460 cells (Lawrence and Thach, 1974).

2.3.3. Preparation of Cell Extracts

The basic method used for the preparation of cell extracts is that described by Mathews and Korner (1970); a similar procedure may be used for all cell types. Tissue culture cells ($1–2 \times 10^9$ cells) are harvested by centrifugation and resuspended in cold isotonic buffer. Ascites cells from five mice, harvested by draining the ascitic fluid from the opened peritoneal cavity into a sterile, precooled beaker, are diluted with cold isotonic buffer (Martin *et al.*, 1961; Mathews and Korner, 1970; McDowell *et al.*, 1972). The ascites cells may be filtered through two layers of cheesecloth as they are collected (McDowell *et*