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THE STRATEGY OF RNA VIRUSES*

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I. INTRODUCTION

IN this presentation I shall contrast three types of animal viruses that in their extracellular state appear very similar, but as replicating viruses are very different. The three types of viruses are: picornaviruses, typified by *poliovirus*; rhabdoviruses, typified by *vesicular stomatitis virus* (VSV), and *RNA tumor viruses.*

The virions of all three of these viruses contain a single strand of RNA which is 2.5 to 4 X 10^6 daltons in length (on the order of 10 kilobases). While these viruses might appear superficially similar because of the similarity of their genome structure, there are enormous differences in the modes of nucleic acid synthesis used by the viruses, the organization of their messenger RNA (mRNA), the types of polymerases involved in replicating them, and the way in which they utilize the hospitality of the host cell.

Although many experimental observations have contributed to our understanding of these viruses, generally a single experiment provided the clue that signaled the unique features of a given virus. In what follows, the key observation for each of the systems will be discussed, and later work on the properties of the virus will then be summarized.

The work from my laboratory described here was often performed by graduate students, postdoctoral fellows, or my close associate Donna Smoler. The names of my other principal associates are: for poliovirus work Michael Jacobson, Charles Cole, Deborah Spector, Martinez Hewlett, Lydia Villa-Komaroff, and Harvey Lodish; for VSV work, Alice Huang, Martha Stampfer, John Rose, David Knipe, and Harvey Lodish; and for RNA tumor virus work, Inder Verma, Amos Panet, and William Haseltine.

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II. POLIOVIRUS

The key observation to our understanding of the life strategy used by the poliovirus genome was the observation many years ago that poliovirus RNA is infectious (Colter *et al.*, 1957; Alexander *et al.*, 1958). At the time that observation was made, it merely confirmed what was known with tobacco mosaic virus, but the further development of animal virology has shown that only a few classes of viruses have infectious RNAs.

The existence of an infectious RNA implies that the RNA polymerase that replicates the RNA is either present in the uninfected cell or is specified directly by the incoming RNA acting as a mRNA. Because no polymerase able to replicate poliovirus RNA has ever been identified in uninfected cells, we have come to believe that at least a critical subunit of the poliovirus replicase is encoded by the viral RNA. A new replicase activity has been identified in the cytoplasm of poliovirus-infected cells (Baltimore *et al.*, 1963)–this is the only evidence for a virus-specific subunit.

Another approach to understanding the poliovirus strategy is to ask whether its virion RNA can act as mRNA. Before showing how we know that poliovirus RNA is a mRNA for the synthesis of the viral proteins, we first must introduce a variant of poliovirus called the poliovirus defective interfering particle, or "polio DI" for short.

Polio DI is a deletion mutant of poliovirus which appeared in our stocks as a contaminant and can be purified away from standard poliovirus because the DI virion has a lower density than the standard virion (Cole *et al.*, 1971). The RNA of DI is smaller than standard RNA based on its migration rate during electrophoresis (Fig. 1), its sedimentation rate in sucrose gradients and competitive hybridization studies (Cole and Baltimore, 1973). Poliovirus makes a double-stranded RNA also called "replicative form" or RF (Baltimore *et al.*, 1964; Baltimore, 1966); an easy way to show that polio DI is a deletion is to measure the length of the standard and DI double-stranded RNAs. From electron micrographs of the double-stranded RNAs, histograms of the lengths of standard and DI RNA have been constructed (Fig. 2). They indicate a difference of 15–20% in the length of the two RNA molecules.

Before turning to the question of whether virion RNA can act as

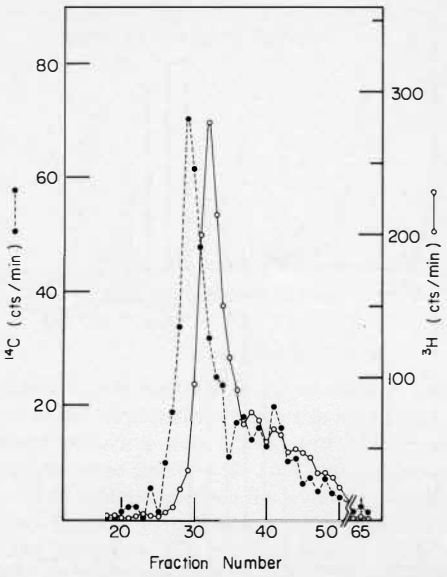

FIG. 1. Comparison of the electrophoretic mobility of standard (●– – –●) and DI (○———○) RNA in polyacrylamide gels. Standard poliovirion RNA labeled with [^{14}C] uridine was mixed with the RNA from DI particles labeled with [^{3}H] uridine. The two RNAs were analyzed by electrophoresis through 2.6% polyacrylamide gels. Migration was from left to right. Reprinted from Cole *et al.* (1971) by permission of the publisher.

mRNA the peculiar nature of poliovirus-specific protein synthesis must be understood. While a large number of discrete polypeptides can be found in the cytoplasm of poliovirus-infected cells, evidence from various types of experiments points to the existence of only a single, long polypeptide (really a polyprotein) as the translation product of the poliovirus genome. This was initially demonstrated in a study of the sizes of the poliovirus proteins made when amino acid analogs replaced certain of the standard amino acids in the growth medium (Jacobson and Baltimore, 1968). A series of long polypeptides were found, the longest of which was the appropriate size to be a total transcript of the poliovirus genome (Jacobson *et al.*, 1970). We have more recently been able to prove that this is a transcript of the whole genome by looking at

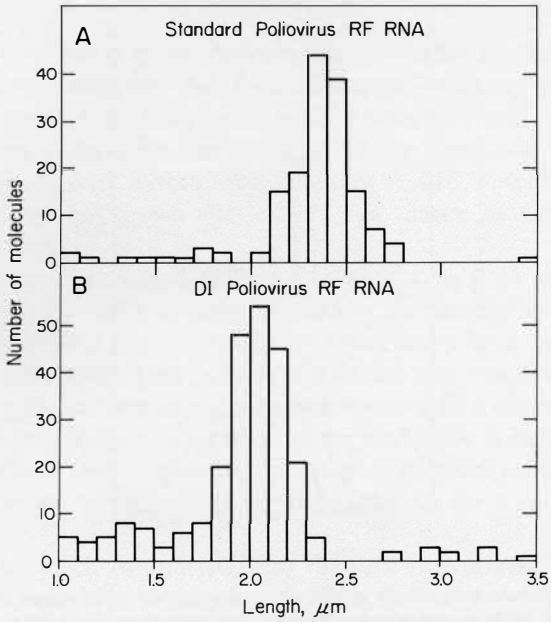

FIG. 2. Comparison of the sizes of double-stranded RNA of (A) standard poliovirus and (B) DI particles. The lengths of poliovirus double-stranded RNA molecules (RF RNA) were determined by electron microscopy. Unpublished data of Dr. M. Hewlett.

the size of this protein in cells infected by standard virus and in cells infected by polio DI (Cole and Baltimore, 1973). Figure 3 represents coelectrophoresis of the virus-specific proteins in cells infected by either standard virus or polio DI and incubated in the presence of amino acid analogs. The largest product (marked NCVP-00) is slightly shorter in the DI-infected cells ("DI(1) NCVP-00") than in the standard virus-infected cells, and calculations indicate that the loss of protein corresponds to about 15% of the total genome (Cole and Baltimore, 1973). Therefore the physical deletion of the RNA and the size of protein deleted are in agreement with each other and thus NCVP-00 would appear to be a translation product of virtually the entire poliovirus RNA.

To show that the poliovirion RNA is able to direct the synthesis of

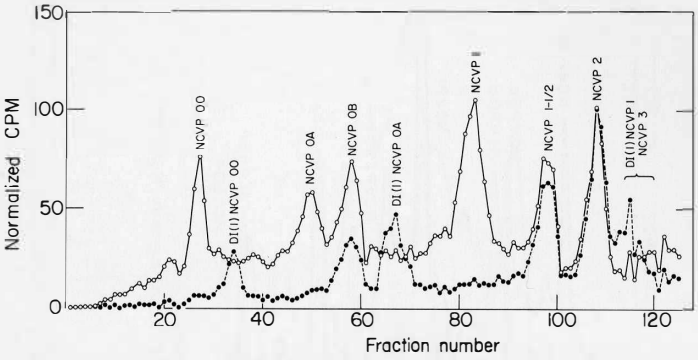

FIG. 3. Comparison of the electrophoretic mobility of virus-specific proteins induced by poliovirus and DI particles in the presence of amino acid analogs. Cells infected by standard poliovirus were labeled with [^{3}H]leucine (○——○); cells infected by DI particles were labeled with [^{14}C]leucine (●– – –●). Both cultures of cells were exposed to medium in which the arginine, phenylalanine, proline, and methionine had been replaced by canavanine, *p*-fluorophenylalanine, azetidine-2-carboxylic acid, and ethionine. The proteins labeled in the two cultures were mixed and analyzed by electrophoresis through 7.2% polyacrylamide gels in the presence of sodium dodecyl sulfate. The protein "NCVP OO" coelectrophoresed with a marker of myosin indicating a molecular weight in excess of 200,000. Migration was from left to right. Reprinted from Cole and Baltimore (1973).

this single, very long polypeptide, use was made of an *in vitro* cell-free system derived from mouse ascites cells. By shifting the salt concentration during a 90-minute incubation from the optimum for initiation (90 m*M*) to the optimum for elongation (150 m*M*), a significant amount of protein of the size of NCVP-00 has been made (Villa-Komaroff, 1975; Villa-Komaroff *et al.*, 1975). When standard RNA was used to program the system, the longest polypeptide product had the same size as the NCVP-00 made in the presence of amino acid analogs (Fig. 4). When the system was programmed by polio DI RNA (Fig. 5), the size of the longest product was shorter than NCVP-00 by the same amount as the longest product made in cells infected by polio DI (compare to Fig. 3). Therefore the same products can be made *in vitro* using a system programmed by virion RNA as are made *in vivo*, and we can conclude that the virion RNA can act as mRNA.

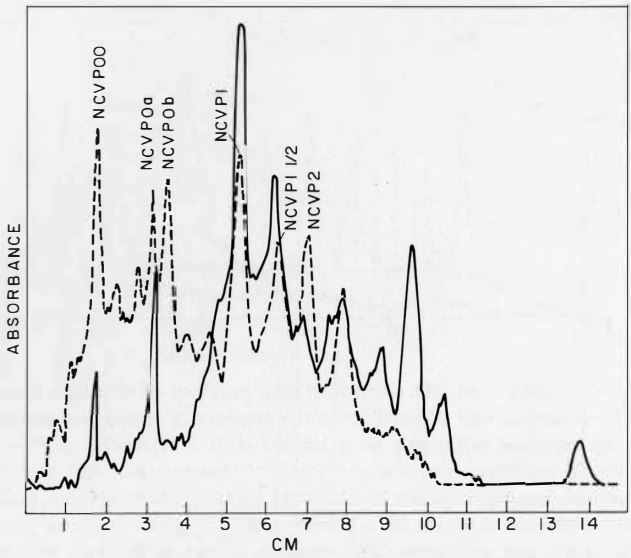

FIG. 4. Polyacrylamide gel electrophoresis of the products synthesized in response to poliovirus RNA in a cell-free extract of mouse ascites cells. The reaction mixture, containing per milliliter 50 μg of poliovirus RNA and 250 μCi of [^{35}S] methionine, was incubated for 15 minutes with 92 m*M* K^+ phosphate and then 105 minutes with 155 m*M* K^+. The products formed were separated by electrophoresis through a polyacrylamide gel and then submitted to autoradiography. The autoradiogram was scanned with a microdensitometer, and the pattern is shown in the solid line (——). In a parallel polyacrylamide gel, the poliovirus-specific proteins made in the presence of amino acid analogs in infected cells were analyzed (– – –). Migration was from left to right. Unpublished data of Dr. Lydia Villa-Komaroff.

Because the two major RNA species needed by poliovirus, the virion RNA and the mRNA, are identical, the replication system of poliovirus need be only very simple. The existing evidence confirms the simplicity of the system because only a few RNA species are involved. Figure 6 shows a schematic representation of the types of RNA molecules involved in poliovirus replication. The incoming "plus" strand synthesizes a "minus" strand, which in turn synthesizes a series of "plus" strands. As we shall see, this simplicity is in marked contrast to the complexity of other viral replication schemes.

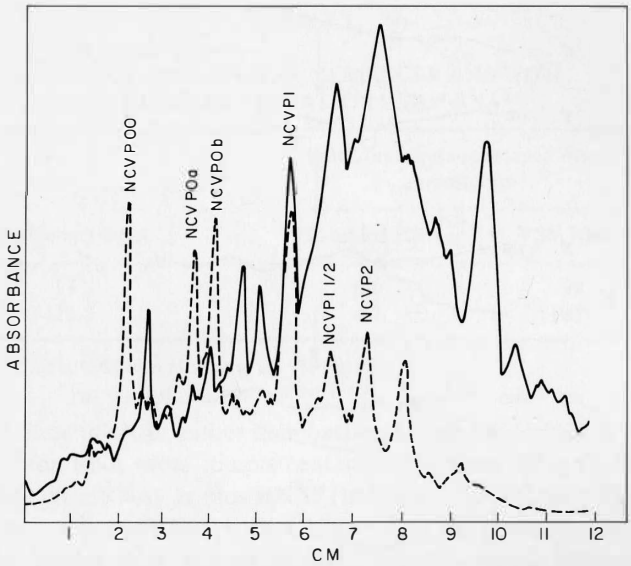

FIG. 5. Polyacrylamide gel electrophoresis of the products synthesized in response to DI particle RNA in a cell-free extract of mouse ascites cells. Methodology as in Fig. 4 except that the cell-free extract was programmed by DI particle RNA. Unpublished data by Dr. Lydia Villa-Komaroff.

An important aspect of the scheme shown in Fig. 6 is the polyadenylic acid [poly(A)] found at the 3′-end of poliovirion RNA (Armstrong *et al.*, 1972; Yogo and Wimmer, 1972; Spector and Baltimore, 1975). It has been shown that the minus strands have poly(U) at their 5′-ends (Yogo *et al.*, 1974), and it appears that the poly(U) encodes the poly(A), and vice versa (Spector and Baltimore, 1975). Cellular messenger RNAs often have 3′-terminal poly(A), and so poliovirus RNA would seem to have appropriated a cellular mechanism. However, in contrast to poliovirus poly(A), cellular poly(A) does not appear to be encoded in cellular DNA, but rather is added to mRNA or its precursor by a posttranscriptional mechanism (Brawerman, 1974).

Poliovirus RNA needs its poly(A) for some critical function: removal of the poly(A) with ribonuclease H plus poly(dT) abolishes the infectivity of the RNA (Spector and Baltimore, 1974). The translation of poly(A)-deficient RNA in a cell-free system is not impaired, however,

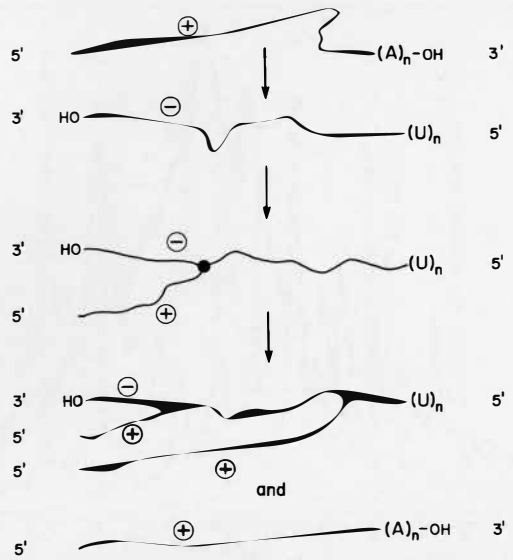

FIG. 6. The poliovirus genetic system. Schematic representation of poliovirus-specific RNA synthesis in the cytoplasm of infected cells.

and so the poly(A) is probably necessary to replication (Spector *et al.*, 1975). Cellular mRNA has never been shown to replicate; poliovirus therefore appears to use its poly(A) for a function not performed in the uninfected cell. This is an interesting example of the appropriation by a virus of a cellular mechanism [the 3′-terminal addition of poly(A)] and its use for a virus-specific purpose.

III. VESICULAR STOMATITIS VIRUS

Vesicular stomatitis virus (VSV) has an RNA genome consisting of a single strand of RNA of about 4×10^6 daltons. The key experiment leading to an understanding of the strategy of VSV was the observation that the mRNA found a polyribosomes was not the 4×10^6 dalton virion RNA, but was rather a collection of smaller RNAs (Huang *et al.*, 1970). Sedimentation of the mRNAs separated them into two size classes: one, a homogeneous RNA sedimenting at 28 S, and the second, a heterogeneous group of RNAs sedimenting with a peak at about 13 S.

TABLE I

ANNEALING OF VIRAL MESSENGER RNA WITH VESICULAR STOMATITIS VIRUS RNA[a]

	% Ribonuclease-resistance when annealed to	
Messenger RNA	No added RNA	VSV RNA
13 S	0.0	99
28 S	0.7	101

[a]Adapted from Huang *et al.* (1970).

Both of these mRNAs, rather than having the same nucleic acid polarity as the virion RNA, were complementary to the virion RNA (Table I). If we define all mRNAs as plus RNAs (Baltimore, 1971), then VSV has a minus strand genome and, in fact, has become the prototype virus for a class of viruses now known as the "negative strand viruses." The discovery that these small RNAs were complementary in base sequence to that of the virion RNA, led to the idea that there might be an RNA polymerase in the virion. Otherwise, it was difficult to understand how infection could start. We therefore assayed disrupted virions for RNA polymerase activity and found that the virion could synthesize RNA (Table II) (Baltimore *et al.*, 1970). Furthermore, the RNA made by the virion would anneal to the virion RNA (Table III). More recently it has been shown that the RNA made by the virion has the same size and structure as the mRNA for viral proteins (Moyer and Banerjee, 1975) and will act as viral mRNA in cell-free systems (Both *et al.*, 1975).

The VSV mRNAs, like cellular mRNAs, have a 3′-terminal poly(A) sequence (Soria and Huang, 1973; Ehrenfeld and Summers, 1972), and one can use this sequence to purify the mRNAs away from ribosomal and transfer RNAs (Rose and Knipe, 1975). Unlike the poly(A) on poliovirus RNA, the poly(A) on VSV mRNAs is not encoded in the minus strand (Marshall and Gillespie, 1972). Rather, a posttranscription addition of poly(A) occurs. The enzyme responsible for VSV poly(A) synthesis is present in the virion (Villarreal and Holland, 1973; Banerjee and Rhodes, 1973).

Using a combination of gel electrophoresis and sucrose gradient

TABLE II

PROPERTIES OF THE VESICULAR STOMATITIS VIRUS RNA POLYMERASE[a]

System[b]	GMP incorporated (nmoles)
Complete	12.4
Minus detergent	0.4
Minus magnesium	<0.2
Minus UTP or CTP	<0.3
Plus ribonuclease	0.5

[a] Adapted from Baltimore *et al.* (1970).

[b] Complete reaction system contained virions of VSV, Tris·HCl, $MgCl_2$, mercaptoethanol, NaCl, ATP, UTP, CTP, ^{3}H-GTP, and 0.1% Triton N-101.

sedimentation, the VSV mRNA population has been separated into four discrete fractions (Rose and Knipe, 1975). There are five proteins in the virions of VSV, and only these five proteins are evident in infected cells (Wagner *et al.*, 1972). The existence of four mRNA classes and five proteins raised the question of which RNAs encode which proteins.

One of the size classes of RNA is the 28 S RNA; this has been shown to encode the largest of the VSV proteins using a cell-free protein-synthesizing system (Morrison *et al.*, 1974). A similar analysis has shown

TABLE III

ANNEALING OF POLYMERASE PRODUCTS WITH VESICULAR STOMATITIS VIRUS RNA[a]

Treatment	% Ribonuclease resistance
Boiled and quickly chilled	4.5
Boiled and annealed with VSV-RNA	94
Boiled and annealed with poliovirus RNA	21
Boiled and annealed with no added RNA	20

[a] Adapted from Baltimore *et al.* (1970).

that two of the three smaller RNA size classes each encodes an individual polypeptide (Knipe *et al.*, 1975; Both *et al.*, 1975). The fourth RNA size class encodes two polypeptides; because this RNA fraction consists of molecules only long enough to encode one of the two proteins whose synthesis it directs, it must consist of two mRNAs that are so similar that they have been inseparable (Knipe *et al.*, 1975; Both *et al.*, 1975). It appears, therefore, that the virion RNA of VSV codes for the synthesis of 5 mRNAs, each of which in turn codes for the synthesis of a single protein. The concept of multiple complementary mRNAs made from a single-input RNA is apparently a general mechanism for many RNA virsues and is not a peculiarity of VSV (Baltimore, 1971; Fenner *et al.*, 1974).

This method of forming the viral mRNAs is very different from that of poliovirus and requires that there be two kinds of VSV-specific RNA synthetic systems rather than the one kind of RNA synthesis which is utilized by poliovirus (Soria *et al.*, 1974; Perlman and Huang, 1973/74). For VSV, the process of *transcription* involves the synthesis of the five individual mRNAs while the process of replication works more or less like the poliovirus replication system. Figure 7 shows these two limbs of the VSV RNA synthetic mechanism; there is extensive evidence supporting this model (Soria *et al.*, 1974).

IV. RNA TUMOR VIRUSES

RNA tumor viruses have an RNA genome which sediments at 60–70 S, a sedimentation rate much larger than that of either VSV RNA or poliovirus RNA. Denaturation of the 70 S RNA, however, causes it to come apart into 35 S RNA molecules, each of which is exactly the same size as poliovirus RNA (see Green, 1970). Recent data from a number of laboratories indicate that all the 35 S RNA molecules of a single virus are identical (see Baltimore, 1975), and therefore the amount of information in the RNA tumor viruses is about the same as that in poliovirus.

In spite of the superficial similarity to poliovirus RNA, the strategy of RNA tumor viruses is very different. The key observation to understanding this system was the realization that DNA plays a significant role in the synthesis of RNA tumor viruses. This concept emerged from work in a number of laboratories during the 1960s (Bader, 1967;

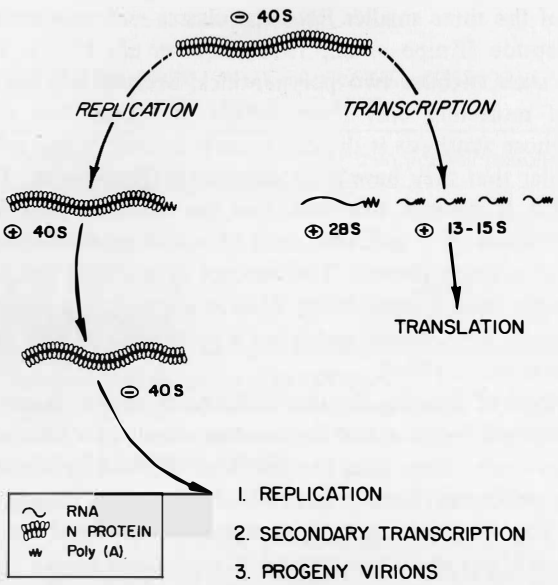

FIG. 7. Vesicular stomatitis virus RNA synthesis in the cytoplasm of infected cells. The 40 S RNA, the genome RNA of the virus, is shown covered by the N protein to form a nucleocapsid. Similarly, its complementary strand, the 40 S plus RNA, is shown covered by N protein as indicated by the data of Soria *et al.* (1974). During VSV transcriptional RNA synthesis, the products are not covered by the N protein but appear to be "free" RNA. The five transcriptional products act as mRNA for the synthesis of the five VSV proteins. Reprinted from Soria *et al.* (1974).

Temin, 1964a,b, 1967, 1970) and was specifically formulated into the provirus hypothesis by Howard Temin (1964a,b). The provirus hypothesis probably received its strongest support from the observation that the RNA tumor virus genome contains an enzyme able to make a DNA copy of the viral RNA (Baltimore, 1970; Temin and Mizutani, 1970; Temin and Baltimore, 1972). This enzyme has been called the reverse transcriptase (Anonymous, 1970).

The reverse transcriptase, like all DNA polymerases, is a primer-dependent DNA polymerase (Baltimore and Smoler, 1971; Smoler *et al.*, 1971; Kornberg, 1969). It is unable to initiate deoxyribonucleotide chains on its own, but rather requires a preformed primer on which to

polymerize the substrate. The enzyme has a number of unique features that distinguish it from cellular polymerases. One of these is the ease with which it copies polyribonucleotides; this is most evident using ribohomopolymers, the most diagnostic of which is poly(C). If poly(C) and an oligo(dG) primer are provided to the reverse transcriptase along with dGTP, the enzyme readily polymerizes dGMP to form poly(dG), and this rapid reaction is virtually diagnostic of the reverse transcriptase (Baltimore *et al.*, 1973; Sarngadharan *et al.*, 1972). Another unique feature of the enzyme is that it contains a ribonuclease H activity (an

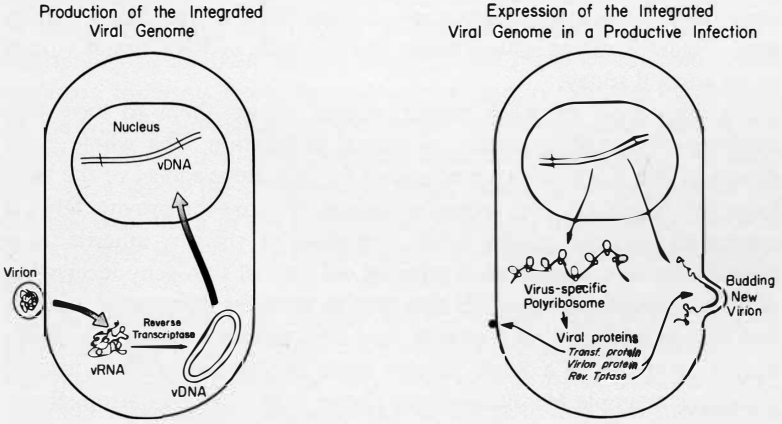

FIG. 8. The life cycle of an RNA tumor virus. Based on the present status of knowledge of the system (Baltimore, 1975), the life cycle of an RNA tumor virus can be separated into two parts. In the first part the virion attaches to the cell and somehow allows its RNA along with reverse transcriptase to get into the cell's cytoplasm. There the reverse transcriptase causes the synthesis of a DNA copy of the viral RNA. Present evidence indicates that a fraction of the DNA can be recovered as closed, circular DNA (Varmus *et al.*, 1974; Gianni *et al.*, 1975), and it is presumably that form which integrates into the cellular DNA. Once the proviral DNA is integrated into cellular DNA, it can then be expressed by the normal process of transcription. The two types of product that have been characterized are new virion RNA and mRNA. Much of the mRNA that specifies the sequence of viral protein is of the same length as the virion RNA, but there may also be shorter mRNAs. The virus-specific proteins have two known functions: one is the transformation of cells that occurs when a sarcoma virus infects a fibroblast, the second is to provide the protein for new virion production.

enzyme activity able to degrade the RNA portion of a DNA–RNA hybrid) (Mölling *et al.*, 1971). No other DNA polymerase is known to contain a ribonuclease H activity, although *Escherichia coli* DNA polymerase I can carry out a similar type of reaction (Baltimore and Smoler, 1972).

A third very unique feature of the avian RNA tumor virus reverse transcriptase has recently come to light. The enzyme contains a specific binding site for $tRNA^{Trp}$ (Panet *et al.*, 1975) and it appears that this binding site is utilized by the enzyme for the initiation of copying of the 70 S RNA (Dahlberg *et al.*, 1974).

The basic strategy of RNA tumor viruses therefore appears to be the utilization of the reverse transcriptase in the virion to make a DNA copy, which can then integrate into cellular DNA and act like a cellular gene. Figure 8 shows schematically the life cycle of RNA tumor viruses as we know it today.

The life cycle of RNA tumor viruses allows them to have two methods of spreading within an animal population. The whole cycle shown in Fig. 8 provides a mechanism for infectious spread of the virus from cell to cell or from animal to animal. Because the proviral DNA is integrated into the cellular DNA, spreading of virus by inheritance is possible. In fact, after a cell is infected, all the cell's progeny receive the virus by inheritance. Animals also receive virus by inheritance, and, in one case at least, the AKR mouse, viral information has been rigorously shown to reside at a single genetic locus and to segregate in genetic crosses as a simple Mendelian locus (Rowe *et al.*, 1972; Chattopadhyay *et al.*, 1974, 1975).

V. CONCLUSION

It is evident that the superficial similarity of the RNAs in the virions of poliovirus, VSV, and RNA tumor viruses is misleading. The three types of viruses use very different strategies for infecting cells. The unraveling of these strategies has come from a series of key observations: the infectivity of poliovirus RNA, the complementary mRNAs of VSV, and the reverse transcriptase of RNA tumor viruses.

The two lytic viruses, polio and VSV, sharply contrast with the RNA tumor viruses. The lytic viruses provide the cell with a completely new system for the replication and transcription of RNA. They establish, in

the cytoplasm of the infected cell, RNA synthetic systems that supply to the cell's ribosomes large amounts of virus-specific RNA. The products encoded by this RNA rapidly take over the cell and as the large quantities of viral products build up in the cell, new virions are formed and the cell dies. The RNA tumor viruses, on the other hand, insinuate themselves into the cell's genetic system and maintain themselves as part of the cell without killing the cell, in fact without seriously perturbing the cell's metabolism.

These two styles of living for viruses, the lytic style and the integrative style, are reflected in the way these two types of viruses maintain themselves in the environment. Polio and VSV must continually infect new animals to propagate themselves. Their life is a neverending cycle of first a few days multiplying in one animal and then a few days multiplying in another animal. Breaking this cycle would completely eliminate the virus from the environment and in fact, the vaccination of the world's population against certain viruses may soon lead to the complete elimination of such viruses from the environment. (We need not worry about the extinction of those viruses, however, because we can maintain them in our laboratories, which are relatively inexpensive and effective zoos for the maintenance of viral genetic information for the future.)

RNA tumor viruses, in contrast to lytic viruses, can use their ability to act as DNA in order to get into the germ line of animals. There they can maintain themselves for very long periods of time without any apparent effect on the animal. Once in the germ line of a species, the viruses will be passed from parent to offspring probably for millions of years. I would guess, although many will disagree with me, that in the germ line of animals many RNA tumor viruses have a negligible effect on the fitness of the animal and therefore are neither selected for nor against by evolution. In such a situation, after many rounds of duplication the viruses will begin to accumulate mutations and it seems evident that unless they submitted themselves to the rigors of evolutionary selection occasionally they would become extinct. Therefore it is not surprising that the viruses can also maintain a horizontal mode of infection under certain conditions, moving from animal to animal just like a lytic virus; in fact, we should not forget that there are lytic viruses that use reverse transcription as a central part of their life cycle (Temin and Baltimore, 1972). Once the viruses have been invigorated

by evolution, they can again go back into the germ line and maintain a covert life for numerous generations. Disturbed only by an occasional mutation, they will be faithfully transmitted from parent to offspring.

ACKNOWLEDGMENTS

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