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Virus reconstitution and the proof of the existence of genomic RNA

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This paper is a historical overview of the work done on the tobacco mosaic virus. The primary finding was that a virus is capable of reassembling itself from its component protein and RNA, and that only the RNA carries the genomic capability of the virus. This was followed by detailed studies of the chemical and biological properties of viral RNA.

Keywords: infectious viral RNA; mutation; translation

During the 1920s, chemists and biologists began to focus their attention on macromolecules. Enzymes, hormones, antibodies, as well as many toxins, were found to be proteins. Methods were needed and were developed to purify, crystallize, and study the structure and function of many proteins. When Wendell M. Stanley isolated and began to characterize a virus by such methods, he believed it to be a protein (Stanley 1935). Only a few scientists were working on DNA, and they found it difficult to characterize this class of macromolecule in chemical terms, although the evidence that DNA was the carrier of genetic information gradually became convincing (Avery *et al*. 1944; Hershey & Chase 1952). Yet, most researchers thought that RNA only played structural roles as polyanionic skeletons holding proteins in their proper structural conformation. This was also our belief when, in about 1950, we joined the Virus Laboratory which Stanley had established two years earlier at the University of California at Berkeley. By then it was known that viruses contained nucleic acids, either DNA or RNA. But most of us did not believe that the small amount of RNA in some plant viruses such as TMV (5%) could have any biological significance.

H.F.-C. had enzymatically coupled amino acids (Bergmann & Fraenkel-Conrat 1938) and crystallized and characterized the first animal neurotoxin, crotoxin, of the Brazilian rattlesnake (Slotta & Fraenkel-Conrat 1938). Thus, our virus work also focused at first on the protein of TMV. Methods for its isolation in native or renaturable form were developed at about this time in Gerhard Schramm's laboratory in Tübingen using weak alkali (Schramm *et al*. 1955), and in our laboratory using acetic acid (Fraenkel-Conrat 1957). The RNA was only considered a by-product of such procedures. Schramm & Zillig (1955) observed that the viral protein was able to aggregate at neutrality into disks and rod-shaped particles resembling the virus, but such rods were not infectious. Furthermore, adding the RNA fraction back to such reaction mixtures had no effect on this protein assembly. In view of the known sensitivity of RNA to alkali this was not surprising. We, however, had previously developed a method of degrading TMV by gentle detergent treatment at neutrality (Fraenkel-Conrat & Singer 1954). It occurred to us that this method, which supplied the pure undegraded, though thoroughly denatured, material for our protein structural studies might represent a source of intact viral RNA. Thus, we repeated Schramm's native-protein aggregation experiments, but added to the reaction mixture the intact RNA obtained by detergent (SDS, sodium dodecyl sulphate) degradation of TMV and fractional ammonium sulphate precipitation. The exciting result was that such reaction mixtures did show viral infectivity when inoculated on host plants (Fraenkel-Conrat 1956*a*,*b*). Electron micrographs showed that such preparations contained many typical 300 nm long TMV rods (Fraenkel-Conrat & Williams 1955). This was in contrast to the aggregates formed by protein alone where the rod lengths ranged from very short to very long. We now know that the length of rod-shaped and fibrous viruses is determined by the length of the RNA molecule which limits and stabilizes the coat protein aggregation. We termed this reconstitution of the virus. The term reconstitution has since come to denote the self-assembly of two (or more) components of biological particles, and TMV may be the first such *in vitro* reassembled particle.

Careful characterization of our 'reconstituted' TMV showed that in all properties the 300 nm long rods were the same as those in the original virus, being much more stable over the tested pH range than the pure protein rods and being, as stated, infectious. While we naturally regarded this as an important finding, we were in no way responsible for statements in the press that this represented 'creation of life in the test tube'.

The yield of full-length rods and of viral infectivity was at first quite low, although conditions were worked out gradually to obtain good yields of reconstitution and infectivity (Fraenkel-Conrat *et al*. 1957). It was obvious that many control experiments needed to be done to prove that the at first quite low infectivity was due to *in vitro*-formed virus rods, since the efficiency of TMV is normally quite low, the number of lesions on leaves being much lower than the number of virus particles applied. One important control was to

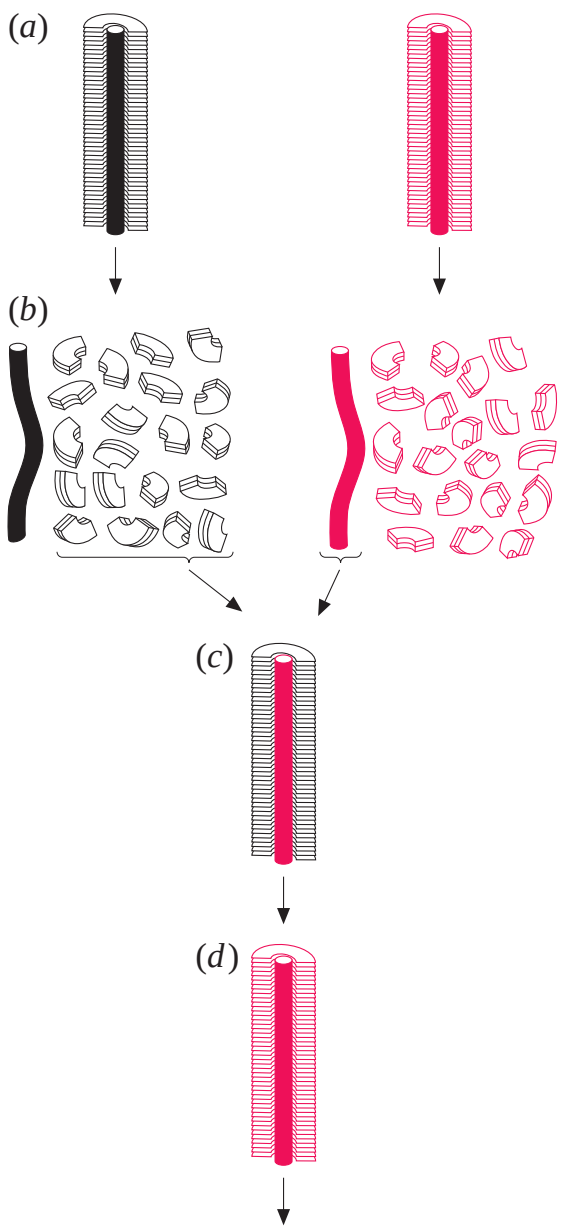

Figure 1. *In vivo* reconstitution of a hybrid virus. (*a*) Shows two strains of virus which were separated into protein and nucleic acid (*b*). The protein of one strain was allowed to recombine with the nucleic acid of the other (*c*). (*d*) The *in vivo* progeny of this hybrid had the protein originally associated with its nucleic acid. Reprinted with permission of *Scientific American* from Fraenkel-Conrat (1956*a*).

inoculate high levels of the protein alone, as well as of the RNA alone, to exclude the presence of residual traces of intact virus in either of these preparations. The protein never caused lesions at any level; the RNA, however, occasionally gave a few lesions at high levels. At this stage H.F.-C. started to attend many congresses and symposia and to give lectures on virus reconstitution. He still was inclined to believe that TMV infectivity required the specific 300 nm rod particle. However, B.S. stayed at Berkeley with the plan to purify the RNA to the level of complete non-infectivity. Unfortunately, or rather fortunately, she was unsuccessful. When she inoculated leaves with newly isolated RNA, obtained after minimal manipulation, it continued to produce lesions, though with only one-thousandth of the efficiency of control TMV (Fraenkel-Conrat 1957). The absence of contaminating virus in such RNA preparations could be conclusively proven by various methods: (i) in contrast to viral infectivity, the infectivity of RNA was not sedimented upon ultra-centrifugation; (ii) RNA infectivity was extremely sensitive to ribonuclease, while TMV was quite unaffected by this enzyme; (iii) antibody to TMV that inactivates it did not inactivate the infectivity of RNA. Gradually, B.S. was forced to conclude that the RNA was infectious *per se* and produced exactly the same disease as intact TMV. Gierer & Schramm (1956) came to the same conclusion at about that time. The low infectivity of the RNA could be attributed to its sensitivity to cellular nucleases when not covered by the large amount of viral coat protein. This was borne out later when the RNAs of other viruses that apparently had icosahedral and less protective protein shells, were found to have a similar level of infectivity to that of the intact viruses.

Another type of experiment, performed concurrently, yielded additional indubitable evidence for the genetic activity and competence of viral RNA. For it proved possible to reconstitute 'hybrid' viruses, combining the protein of wild-type TMV with the RNA of a strain, then termed Holmes ribgrass (HR) strain, that differed from the wild-type or common TMV in the symptoms it produced on plants and, most importantly, also in its coat protein. HR has two amino acids, histidine and methionine, which common TMV lacks. When HR RNA was reconstituted with common TMV protein, and inoculated onto a suitable host plant it produced the symptoms characteristic of HR infection. When progeny virus was then isolated and analysed, its coat protein had HR's composition, characterized by the presence of the two amino acids that the coat protein of the inoculum, comprising 95% of its mass, lacks (Fraenkel-Conrat & Singer 1957).

It had thus been proven beyond a doubt that RNA represented the genetic component of RNA viruses, a finding even more important than the preceding one, that simple viruses can be reconstituted *in vitro*. Such viral RNAs of definite molecular weights of *ca.* 2×10^6 Da were much easier to characterize and study in chemical terms (homogeneity, monodispersity, molecular weight, end groups, nucleotide sequences, etc.) than any DNA of then known viruses, or cells. They represented a most useful object for the study of nucleic acid structure and function. Such viral RNAs served for a decade as the prime research material in the development of molecular biology as coding, mutagenesis, translation and protein synthesis came to be understood. Here are a few examples of the uses of TMV RNA in the 1960s and 1970s.

(1) When Marshall Nierenberg had perfected his *in vitro* translation system, he needed a natural mRNA with a known translation product. TMV RNA and its coat protein came to mind and he came to our laboratory to do the necessary experiment. While he was there, there came the dramatic phone call from his collaborator at their NIH laboratory, J. H. Matthaei: PolyU was translated to polyphenylalanine, the first

result towards understanding the code that related nucleic acids and proteins. Concerning the TMV RNA translation experiments, no coat protein could be detected, and it took 15 years before Knowland's laboratory (Hunter 1976) could solve that problem. The coat protein gene, located at the 3′ end of the viral RNA, could be translated only after it was *in vivo* or *in vitro* released and freed from conformational restraints.

(2) When the biological activity of RNA was discovered, much of the research focused on its chemistry. As stated, the infectivity depended on the integrity of the RNA molecule. A single break, caused by a nuclease, alkali, or other means, in the 6490 nucleotide chain caused loss of infectivity. Many subsequent studies of the action of various non-degrading chemicals showed that all of them caused more or less loss of infectivity. However, some of these modification reactions caused the appearance of mutants among the surviving molecules. This was first reported from Schramm's laboratory (Gierer & Mundry 1958; Schuster 1960) as a consequence of nitrous acid treatment, which besides other effects caused obvious mutagenic events such as C→U, G→X (xanthine) and A→HX (hypoxanthine) transitions. The mutagenicity of hydroxylamine and methoxyamine was similarly accounted for by the change in the predominant tautomeric state of the resultant 4-hydroxy- or 4-methoxy-amino C to resemble U (Singer & Fraenkel-Conrat 1974). Although most alkylating agents are poor mutagens, it was (and remains) surprising that N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was very highly mutagenic when acting on the virus, but not when acting on the RNA (Singer 1968).

Hundreds of TMV mutants were subsequently obtained in Berkeley (California), Tübingen, and elsewhere. The coat proteins of these mutants, the only easily accessible gene product of plant viruses, were then analysed for amino-acid composition, and some for partial sequences. Many mutants showed no changes in the coat protein, which is not surprising since it represents only *ca*. 8% of the total potential gene product. When mutants did show one to three amino-acid replacements, these were considered in terms of the codon dictionary that was being deciphered at the same time in Nierenberg's and Ochoa's laboratories. This led to frequent phone calls from these laboratories to ascertain whether our mutations fitted their developing code assignments. Most of the exchanged amino acids could be attributed to the C→U or A→HX nucleotide exchanges that could be expected from chemical modification of the RNA. Thus, prolines (C,C,C) were often replaced by leucines and serines (C,C,U), and these were in turn replaced by phenylalanines (C,U,U), all well accounted for by the C→U exchanges that mutagens such as nitrous acid would cause. Of special interest was a particular mutation that changed the proline near the C-terminus to leucine (Tsugita & Fraenkel-Conrat 1960). It is the presence of that proline that prevented carboxypeptidase from degrading TMV protein beyond the C-terminal threonine. Harris & Knight (1952) would have obtained a confusing multitude of amino acids released by carboxypeptidase, rather than the single threonine per chain, if they had used such a mutant instead of wild-type TMV. This location of a proline near the C-terminus, as well as the acetyl group on the N-terminus (Narita 1958), probably represent favourable evolutionary developments in producing a virus coat resistant to exopeptidases. The tight folding of the peptide chain actually makes the virus surprisingly resistant also to endopeptidases and proteases.

In conclusion then: TMV supplied the first material enabling the study of biological particle assembly; it supplied the first evidence for the genomic capability of RNA; and it permitted, for the first time, mutagenesis to be accounted for in specific chemical terms.

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