Proceedings of the Second International Congress for Virology
Budapest 1971

International Virology 2

Edited by Joseph L. Melnick, Houston, Texas

8 figures

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Second International Congress for Virology

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Preface

The Second International Congress for Virology, held in Budapest, Hungary during the week of 27 June–3 July 1971, was the first formal meeting of the Virology Section since its official formation under the auspices of the International Association of Microbiological Societies (IAMS). In attendance were approximately 1,000 research virologists representing more than 40 different countries, a wide cross-section which made possible the invaluable exchange of latest developments and activities.

Dr. Zoltán Szabó, Minister of Health of the Hungarian People's Republic and Honorary President of the meeting, welcomed the Congress to his country, emphasizing the significance of virology in the world today.

In remembrance of Dr. John R. Paul and Dr. Wendell M. Stanley, the Secretary-General recognized the great contributions which these men had made over many years to the development of the field of virology. Both men had been invited to speak at this Congress, but illness and untimely death intervened.

Included in the Scientific Program of the Congress were five plenary sessions on topics of general interest to all virologists and thirty specialized workshops from which the participants selected those which they wished to attend. The spectrum of topics offered for discussion ranged from virus-controlled information to viral epidemiology and recent advances in such areas as water research, plant virology, and human virus infections. The program was originally designed to place primary emphasis on current and ongoing research and concepts, so that this material could be assimilated and new ideas be forthcoming; it is hoped that the program did move towards accomplishing this end.

This Congress was the first to meet within the official framework of the

IAMS. The Council of the Virology Section met in Budapest and adopted the statutes by which the organization will function. These are set forth elsewhere in this volume. In addition, the Council also discussed and approved the creation of an international journal to be administered by the Virology Section.

Grateful acknowledgements are due to Dr. György Berencsi in Budapest for his many contributions towards organizing the Congress. We also thank Verle Rennick, Joyce Borak, and Wanda Niebuhr in Houston, and Ilona Koch, Agnes Schwanner, and Maria Winkler in Budapest, for their able and untiring assistance in the preparations for the Congress and the editing of these Proceedings.

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Session 1

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Viral Nucleic Acids

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The RNA-containing coliphages are classified into three serological groups. The first of these comprises phages such as f2, R17 and MS2, which are quite similar among themselves and differ considerably from Qβ, a member of the third group. These four phages have been studied intensively in the last

few years. Their RNAs consist of about 3,500 nucleotides and comprise 3 cistrons. The capsid consists of about 180 coat protein subunits and at least one molecule of a further virus-specific protein, the A or maturation protein (Qβ contains a further protein, designated A_1, which may arise by read-through from the coat cistron into the subsequent nucleotide sequence). The third viral cistron codes for the β-subunit of the viral replicase. The cistron order is A-coat-replicase for both phage groups. The complete amino acid sequence of the different coat proteins has been established; only the first few amino acids of the A proteins and the replicase subunits are known.

The nucleotide sequences of the phage RNAs are being studied in several laboratories. The strategy currently applied may be broken down into three phases: (1) reduction of the viral RNA into subsets of about 50–200 nucleotides in length; (2) determination of the nucleotide sequence of the subsets; (3) alignment of the subsets in their natural order. Only the first of these steps are discussed below.

I. Structure and Function of Phage RNA

Two principal approaches have been used to obtain RNA segments susceptible to nucleotide sequence analysis. The degradative methods are based on the use of nucleases under conditions where only a limited number of phosphodiester bonds are split. Relative resistance to nucleolytic cleavage is inherent in the secondary and possibly tertiary structure of the RNA, since linkages between nucleotides located in double-helical regions are less susceptible to enzymatic attack than those present in single-stranded regions. The isolation of pure RNA fractions from the mixture of degradation products is one of the main technical bottlenecks. Two main separation techniques have been developed for this purpose, namely gel electrophoresis and displacement chromatography. FIERS *et al.* (Belgium) reported on a *system of two-dimensional acrylamide gel electrophoresis*, with the first dimension at pH 3.5 in the presence of urea and the second at pH 8, in a more tightly cross-linked gel, which gives a very high resolution. JEPPESEN *et al.* (UK) have further developed a *separation technique* consisting of electrophoresis on cellulose acetate at pH 3.5 in the first dimension, and of displacement chromatography on DEAE thin layers (homochromatography) in the second. This method allows the separation of large oligonucleotides in a reproducible fashion, thereby providing characteristic fingerprints of a viral RNA. Small differences in the nucleotide sequences of the RNAs of f2, MS2 and R17 have been detected by this method.

Specific protection of certain regions of viral RNA against nucleases can be attained by binding ribosomes to the RNA. This procedure has allowed the isolation of the RNA segments corresponding to the ribosome-binding sites of all cistrons of R17 as well as of the coat and the A cistron of Qβ. A similar approach, in which Qβ replicase was bound to Qβ RNA prior to digestion with T_1 RNase has yielded a piece of RNA containing a binding site for the viral polymerase.

A synthetic approach to the preparation of viral RNA subsets was reported on by BILLETER *et al.* (Switzerland). Qβ RNA is synthesized *in vitro* by Qβ replicase, using either a plus or a minus strand as template. Synthesis begins with the 5′-terminus and proceeds with an elongation rate of about 6 nucleotides/sec at 20 °C. By synchronizing RNA synthesis and using α-^{32}P-labeled ribonucleoside triphosphates as substrates during defined periods of synthesis it becomes possible, in principle, to synthesize either plus or minus strands radioactively labeled in any desired segment. In practice, however, synchrony diminishes as synthesis proceeds, so that the method has so far been utilized only to examine the first few hundred nucleotides at the 5′-termini of both plus and minus strands. A new approach now reported by the Zurich group allows resynchronisation of synthesis at an interior position of the RNA. A ribosome is bound to the coat cistron binding site (the only site ribosomes bind to in Qβ RNA) and the RNA-ribosome complex is used as a template for Qβ replicase, with unlabeled ribonucleoside triphosphates as substrates. Elongation ceases when replicase reaches the ribosome. The ribosome is then removed from the RNA by treatment with EDTA and the replicating complex is separated from substrates and EDTA by chromatography on Sephadex. On addition of radioactive substrates, synchronized synthesis ensues and a labeled minus strand segment is produced, which extends from the region complementary to the coat initiation site into the region complementary to the end of the A protein cistron.

In the case of the RNA of phage group I (using data obtained both from R17 by the Cambridge group and from MS2 by the Ghent group) three main segments have been elucidated. The first extends from the 5′-terminus into the beginning of the A cistron (145 nucleotides), the second from the intercistronic region after the A cistron through the coat cistron (with a short stretch of 16 nucleotides still unknown) and into the beginning of the replicase cistron (about 420 nucleotides) and the third comprises the last 70 nucleotides of the molecule. In total, about 650 nucleotides, or almost 20% of the genome has thus been elucidated.

In the case of Qβ, the two main segments known at present extend from the 5′-terminus to the 330th nucleotide (located well within the A cistron), and from the 160th to last nucleotide to the 3′-terminus. Moreover, a segment of 50 nucleotides corresponding to the intercistronic region between A and coat cistron and extending into the beginning of the coat cistron is known. Thus, 540 nucleotides or 15% of Qβ RNA have been elucidated.

Large parts of the sequences elucidated can be folded to give extensively hydrogen-bonded hairpins, using rules such as those advanced by TINOCO *et al.* In the case of the MS2 coat cistron 64% of the nucleotides are believed to take part in such secondary interactions. The proposed structures are supported by the finding that, under conditions of partial digestion, nucleolytic splits are introduced almost exclusively into single-stranded regions, and into loops and bulges rather than into stem regions of the proposed hairpins.

Since recombination does not occur among RNA phages, the order of their cistrons could not be elucidated by genetic techniques. This information was finally obtained by chemical techniques, in principle by searching for nucleotide sequences corresponding to the beginnings and ends of the phage proteins and locating the absolute or relative positions of these nucleotide sequences on the RNA strand. In all cases, the order of cistrons is A-coat-replicase. Extended non-coding nucleotide sequences are located at the beginning (62 nucleotides in Qβ, 129 in the MS2-R17 group) and the end (at least 32 in Qβ and not less than 10 in the MS2-R17 group) of the RNAs, as well as between the cistrons (for example, 36 nucleotides between coat and replicase cistron of R17).

Since the sequences of amino acids of R17 coat protein and that of the nucleotides in the corresponding cistron are known, it is possible to deduce, at least in part, the genetic code independently of previous data. All assignments made in this fashion agree with the Nirenberg-Ochoa-Khorana code. In addition, it was found that for certain amino acids not all degenerate codons are used in the translation of the coat. For example, AUU and AUC are each used 5 times for isoleucine, while AUA is not used at all; tyrosine is coded for 4 times by UAC, and never by UAU.

The nucleotide sequences around the initiation sites of five different cistrons are now known (all cistrons of the R17-MS2 group as well as coat and A cistron of Qβ). Initiation of phage cistrons always occurs at an AUG triplet, although GUG is also an efficient initiator codon *in vitro*. No extensive sequences are common to all initiation sites; however, certain short sequences are found prior to the initiator AUG of some cistrons (for example, GGUUUGA occurs prior to both the A and the coat cistron of R17, and

UUUGA before the Qβ coat cistron; UAAGAGGA precedes the A cistron of Qβ, UAGGAGG the A cistron of R17 and GAGGA the replicase cistron of R17). If recognition of a determined nucleotide sequence rather than some 3-dimensional structure is required for ribosome binding it would seem that more than one such sequence is possible. Another remarkable aspect of ribosomal binding sites is that some are directly available (the coat cistron binding sites in both R17 and Qβ) while others are cryptic, i.e., not available on the mature, native RNA strand (for example, the A cistron-binding site of Qβ which only binds ribosomes detectably when present on an RNA fragment or on a nascent strand). It is thought that this unavailability of the binding site is due to the secondary and/or tertiary structure of the RNA, and in at least one case tentative structural evidence is forthcoming to support this view. It had been shown that ribosomes do not initiate polypeptide synthesis at the replicase cistron of native viral RNA unless the coat cistron was being translated. It was suggested that an interaction between the replicase initiation site and a region of the coat cistron prevented ribosome attachment to the former. Translation of the coat cistron was thought to be instrumental in reversing this interaction, thereby allowing translation of the replicase cistron. FIERS and his colleagues have now pointed out that a complementary relationship in fact exists between an RNA segment (nucleotides 72 to 96) of the coat cistron and the initiation site of the replicase cistron, lending strong support to the hypothesis described above.

As first noted by SPIEGELMAN, the viral replicases show a very high template specificity for the homologous intact RNA, to the exclusion of all other, unrelated viral RNAs and most other RNAs examined. It has been suggested that the non-translatable regions at the termini of the viral RNAs are responsible for the recognitive interaction with the replicase, and some evidence has been offered that the integrity of the 3′-terminus (up to the penultimate nucleotide) is required for template function. However, this cannot be the only requirement, since Qβ RNA fragments (comprising the 3′-terminal fragment) with an average chain length of several hundred nucleotides do not elicit initiation of RNA synthesis. It has been observed that Qβ replicase binds strongly and specifically to Qβ RNA. This binding entails protection of a segment of the RNA against nuclease digestion. Analysis of the protected RNA has revealed that it is derived from the intercistronic region between A protein and coat cistron and consequently comprises part of the ribosome-binding site of the coat cistron. This recognition, therefore, occurs at a position one third strand length removed from the 5′-terminus. It is not clear as yet whether this interaction is required for RNA chain initiation (cf. below).

After invading its host, the phage RNA serves first as messenger for phage-specific protein and subsequently as template for its own replication. During the first process the viral RNA is present as a polysome, with ribosomes traveling in the 5′- to 3′-direction. In replication, the viral polymerase advances along the template in the 3′- to 5′-direction, i.e., on a collision course with translating ribosomes. Since replicase cannot dislodge ribosomes bound to Qβ RNA, a special mechanism must exist to free the parental RNA of ribosomes and render it competent as template for replication. It was found that Qβ replicase strongly and specifically inhibits binding of ribosomes to Qβ RNA while allowing translating ribosomes to terminate synthesis and detach. Since ribosome attachment does not occur at the A cistron of the mature RNA, and initiation at the replicase cistron is dependent on translation of the coat cistron, this process ultimately leads to a dismantling of the polysome. As mentioned in a previous section, an RNA segment protected by Qβ replicase against RNase digestion was isolated and its nucleotide sequence determined. It was found that this replicase binding site was derived from the intracistronic region between the A and the coat cistron, comprising the left half of the ribosome binding site. This finding provides a molecular basis for the repressor activity of Qβ replicase.

Phage Qβ differs substantially in many regards from phages of group I, such as R17 and MS2. Although competition hybridization had not disclosed any relationship between the RNAs of the two phage groups, several significant homologies have since been found in their nucleotide sequences. Also the coat proteins of the two groups show remarkable similarities, so that a common RNA phage ancestor has been postulated.

Comparison of the nucleotide sequences of the coat cistrons of the closely related phages R17 and MS2 by FIERS *et al.*, has revealed that although the coat proteins differ by only one amino acid, there are at least 9 nucleotide changes, i.e., one for every 43 nucleotides. By contrast, the nontranslatable regions show no differences in their nucleotide sequence. The cistronic regions are thus subject to genetic drift, inasmuch as their phenotypic expression can be maintained unaltered by virtue of the degeneracy of the genetic code. As pointed out by FIERS, base changes are also such that virtually no changes in the secondary structure are to be expected. The as yet unknown function of the extracistronic regions is apparently stringently dependent on the primary structure of the RNA.

Despite its apparent simplicity, the replication of RNA phages entails a number of sophisticated control mechanisms. Since the amount of genetic information encoded in the phage genome is limited to three cistrons, evol-

utionary pressure has imposed more than one function onto each of the phage components. The coat protein is not only a structural component of the particle, but serves to modulate translation of the viral genome; the replicase, endowed with a remarkable template specificity, not only distinguishes viral plus and minus strands from virtually all of the other RNAs present in the host cells, but also fulfills the role of a translational repressor, a function essential in the early stages of viral replication. The viral RNA has evolved within the narrow constraints imposed by the multiple demands made on it (a) as a genome which is to be replicated, compactly folded and encapsidated in a symmetrical particle and (b) as a messenger subjected to translational control and moreover required to retain its integrity in an aggressive environment. The hope of eventually understanding this plethora of functions at the molecular level sustains those engaged in the arduous task of elucidating the structure of the phage components.

II. Chemical Modifications of Nucleic Acids

Two papers dealt with chemical modifications of nucleic acids. TIKCHONENKO (USSR) has used O-methylhydroxylamine and formaldehyde to study the *reactivity of the double-stranded DNA inside phage particles*. An appreciable fraction of that DNA showed an altered (not typically double-stranded) secondary structure, which did not correspond to the denatured state, but involved close approximation of protein and DNA. This was supported by the fact that protein amino groups substituted the amino group of cytosine residues after these became activated by addition of CH_3-ONH_2 to the 5,6-double bond.

FRAENKEL-CONRAT (USA) discussed the chemical modification of TMV-RNA and of poly-C, and the *chemical basis for mutagenicity* as tested biologically and by altered template properties, respectively. Hydroxylamine and methoxyamine were found to act as mutagens in both systems through replacement of the amino group of cytosin by RO-NH-(R = H or CH_3); nitrosoguanidine and other alkylating agents acted as mutagens through methylation of the N-3 position of cytosin. This modification (in contrast to the above reaction and deamination) is nonspecific as far as base recognition is concerned. Finally the effect of bromination was discussed which also makes cytosin behave like uracil, though the mechanism of this effect is not attributable to the chemically identified results of the bromine treatment.