

Positive-Strand RNA Viruses

Among the animal viruses, the major families of (+) strand RNA viruses include:

Picornaviruses
Togaviruses
Flaviviruses
Caliciviruses
Coronaviruses

Common features of (+) strand RNA viruses

- 1) replicate in the cytoplasm
- 2) genomic RNA is non-segmented
- 3) genomic RNA's are infectious and have messenger activity
- 4) viruses do not contain any RNA or DNA polymerase enzymes
- 5) all make polyproteins which must be processed
- 6) we can easily distinguish between structural and non structural proteins
- 7) nonstructural proteins associated with at a minimum the following activities:
RDRP, RNA helicase, protease
- 8) most contain multiple copies of only 3 or 4 different structural proteins. The coronaviruses are more complex because of their larger size.
- 9) have an icosahedral structure. The coronaviruses are an exception because their nucleocapsid is helical, but they do appear to contain an icosahedral inner core.

Retroviruses are "positive-strand RNA viruses", but since their replication strategy is completely different from the viruses listed above, they are not grouped with the (+) strand RNA viruses.

Most, but not all plant viruses are RNA viruses, and have a (+) strand RNA genome.

Family Picornaviridae

genome about 7.5 kb, and includes the following genera;

enterovirus	rhinovirus
hepatovirus	paraechovirus

aphthovirus cardiovirus

Viruses in the genera aphthovirus and cardiovirus cause disease in cattle and mice respectively.

Family Flaviviridae

genome about 10-11 kb, and there are 3 genera

genus flavivirus: These are mainly arthropod-transmitted

genus pestivirus: bovine viral diarrhoea virus, BVDV

genus hepacivirus: hepatitis C virus, HCV

Family Togaviridae

genome about 11-12 kb, and there are two genera

genus alphavirus: these are mainly arthropod-transmitted

rubivirus, rubella virus

Family Coronaviridae genome about 27-32 kb

Family Caliciviridae genome about 7.5 kb

Some comments on these different virus families:

- 1) The Togaviridae, Flaviviridae, and Coronaviridae are enveloped viruses.
Picornaviridae and Caliciviridae are naked viruses.
- 2) Genes for structural proteins can be located 5' or 3' in the genome.
If there is a single ORF, the genes are 5' (Picornaviridae and Flaviviridae).
When there is more than one ORF, the genes for the structural proteins are 3' (Togaviridae and Coronaviridae).
The Togaviruses and Caliciviruses make subgenomic mRNAs, and the Coronaviruses actually make a nested set of mRNA's.
What are the implications of having more than one ORF for the regulation of viral protein synthesis?
- 3) Infectious clones have been generated for Picorna- Toga- and Flaviviruses.
These are plasmids that contain a cDNA copy of the entire viral RNA genome downstream of a phage promoter, e.g. T7. When these plasmids are linearized and incubated with the appropriate phage RNA polymerase, RNA transcripts are made which are infectious, i.e. when they are transfected into cells, the cells make infectious virus progeny.

Family Picornaviridae (poliovirus is the prototype)

Among animal viruses, many notable firsts were achieved with picornaviruses, primarily with poliovirus.

- 1) studies of viral RNA synthesis and the demonstration of replicative form (RF) and replicative intermediate (RI) species of viral RNA
- 2) demonstration of an RDRP activity
- 3) the demonstration that viral proteins are synthesized as a polyproteins, which are then processed into many individual proteins.
- 3) RNA recombination
- 4) first viral structures at the atomic level (among animal viruses)
- 5) in vitro synthesis of infectious virus

Properties of poliovirus

1: an icosahedral structure: a solid geometric form with 20 sides and 12 vertices

2: Small, about 30 nm. or less in diameter

3: non-enveloped or naked

4: RNA genome: (+) strand, non segmented

Poly A at 3' end, genetically encoded.

No cap at 5' end, instead there is a small peptide, VPg, 22-24 amino acids in length, covalently linked to the 5' end of the viral RNA.

VPg is covalently bound to 5' terminus of RNA via a Tyr. residue (3rd residue from N terminus).

VPg is on all nascent RNA molecules, and on all viral RNA molecules, (positive and negative strand) except for polysomal-associated or viral mRNA.

5' terminus of viral RNA is pUp

VPg serves as a primer function in RNA replication

To generate viral mRNA, VPg is removed, probably by a cellular enzyme assoc. with polysomes.

5: 4 structural proteins: VP1, VP2, VP3, and VP4. VP4 is internal.

A quick overview of replication

Replication of poliovirus is very rapid, and is characterized by severe CPE.

Steps in replication

1) virus entry : binding and entry. A specific receptor has been identified; it will be discussed later. Virus lacks an envelope, but somehow the virus or at least the viral genome must traverse the cellular membrane.

2) viral RNA gets into cytosol, where it quickly associates with ribosomes and is translated into a single polyprotein which is processed or cleaved into multiple end products.

3) certain of the viral translation products, mainly the RNA-dependent RNA polymerase (RDRP), then serve to

- use (+) strand as template to synthesize (-) strand RNA.

- then to use (-) strand as template to synthesize (+) strand RNA.

- (+) strand RNA is made in great excess over (-) strand RNA. Probably by a ratio of 50:1.

4) What are the possible fates of newly synthesized positive strand RNA?

The viral **replicative intermediate or RI** RNA has 6-7 nascent strands on the RNA template and represents the structure on which RNA molecules are being synthesized.

Infected cells also contain full length double- stranded RNA molecules which are referred to as **replicative form or RF** molecules.

5) When the viral structural proteins reach a critical concentration, there begins a process of **self assembly**, first into subunits, then into viral particles.

- This involves a series of ordered steps.

- 5s subunits contain 1 copy each of VP0, VP1, and VP3; these 5s subunits are called protomers.

- 5 of the 5s particles form 14s particles or pentamers. These contain 5 copies of each of the above proteins.

- Twelve pentamers come together to form 75s icosahedral procapsids; these are empty, i.e. they contain no RNA

- These procapsids contain 60 copies of VP0, VP1 and VP3

- The 125s particle, the provirion, contains viral RNA, but VP0 is still uncleaved.

- In the mature, 150s, infectious particle, VP0 has been cleaved to VP4 and

VP2.

The details of how the RNA is packaged is still largely unknown

Synthesis of Viral Proteins

In eukaryotic cells there is generally one site on an mRNA molecule for initiation of translation, and it is not necessarily the first AUG,

Contrast with prokaryotes.

Thus in eukaryotic cells, One mRNA gives one polypeptide chain.

With poliovirus RNA

there is one initiation site.

The RNA genome contains a single long ORF; therefore one long polyprotein is made from which all the viral proteins are derived. Although the poliovirus RNA gives rise to many proteins, they are all processed or derived from a single polyprotein.

Primary cleavages give rise to P1, P2, and P3, and secondary cleavages to the end products

The viral structural proteins

VP1, VP2, VP3, and VP4

VP0 is the precursor of VP4 and VP2.

Nine of the cleavages are between Gln and Gly residues, but cleavage does not occur between every Gln/Gly pair. Therefore the surrounding amino acid residues, and/or the 3-dimensional structure of the protein must also be important in determining the cleavage sites.

Implications of this type of processing strategy

All viral proteins must be made in equivalent amounts.

There can be little or no differential synthesis of the viral proteins or temporal control over protein synthesis.

More about processing of viral polyproteins

Polyproteins are characteristic of 3 of the families of viruses (Picorna, Flavi, and Toga-viridae) we are discussing.

Polyproteins must be cleaved at very specific sites.

With Togaviridae and Flaviviridae, certain of the cleavages are carried out by cellular proteases, such as signalases. The proteins processed by cellular proteases are viral proteins which are membrane-associated. Other cleavages are carried out by virus-encoded proteases.

With polio all cleavages are carried out by viral-coded proteases, of which there are two.

Viral proteases are also important because in some cases they have proved to good targets for antiviral chemotherapy.

Which poliovirus-encoded proteins have protease activity?

The two poliovirus proteases are the proteins, 2A, and 3C.

2A carries out only one cleavage, between P1 and P2.

All the other cleavages are carried out by 3C.

The primary cleavages are all in cis, whereas the secondary cleavages are in cis or trans.

Both proteases are active in the nascent protein strand and thus can cleave themselves and thus can be released by self-cleavage.

Actually both 3C and 3CD have protease activity, but with somewhat different specificities. e.g. the secondary cleavages of P1 are carried out by 3CD, not 3C.

Why must viruses encode proteases rather than use cellular proteases?

1. for the desired specificity
2. need for the protease activity at specific locations in the cell, i.e. where viral proteins are being made.

The polio polyprotein and the cleavage cascade

1. P1/P2 Co-translationally by 2A^{pro} Y/G
2. most of the cleavages By 3C^{pro} Q/G
3. A maturation cleavage, VP0 to VP4 and VP2 seems to occur after association of viral RNA with protein shell, but have not identified the enzyme that does this cleavage.

2A^{pro} and 3C^{pro} are both cys proteases

The activity of both these proteases is inhibited by cpds which affect SH groups, consistent with idea that the active site contains an -SH group. But they fold similarly to chymotrypsin-like enzymes which are serine proteases.

The catalytic triads are

for 2A-pro H-20 D-38 C-109

for 3C-pro H-40 D-71 C-147

Poliovirus infection is characterized by rapid shutoff of host macromolecular synthesis

Evidence that one of the viral proteases, 2A, cleaves a host protein, eIF4G, which is important in cap recognition and initiation of protein synthesis. Thus poliovirus subverts the cell's protein synthetic machinery and converts it entirely to its own

use.

The synthesis of poliovirus proteins does not require cap recognition.

Translation of PV RNA: mechanism of initiation

PV RNA has an unusually long 5' untranslated region (UTR), 742 nt or 10% of the genome.

In the UTR there are about 8 AUG codons which do not function as initiation codons, before the actual AUG initiation codon.

According to the scanning model for initiation of protein synthesis the initiation complex binds to the 5' cap structure, then scans the RNA for first AUG in suitable context.

But PV RNA has no 5' cap structure.

Instead PV RNA has in its 5' UTR an **Internal Ribosome Entry Site or IRES**

The IRES is a region in the RNA about 420 nt in length, with very significant secondary structure, and it promotes initiation of protein synthesis by internal ribosome entry, which is a cap-independent mechanism for initiating translation.

Various host proteins involved with protein synthesis interact with different domains of the IRES.

The idea of internal initiation of protein synthesis, as opposed to the scanning mechanism, generated considerable controversy at first, but now is accepted.

An experiment which demonstrates internal initiation mediated by an IRES: Making use of a PV infectious clone, an EMC (another picornavirus) IRES was inserted between the P1 and P2 coding regions of PV RNA, and a termination codon was placed at the end of the P1 region. This cDNA sequence was incorporated into a T7 transcription vector.

Also made use of a T7 vector with normal PV cDNA as a control.

Made transcripts from both vectors and translated them in HeLa cell lysates.

Both transcripts gave similar pattern of proteins

Both transcripts produced virus when transfected into HeLa cells and both transcripts produced CPE.

RNA from the virus with the inserted IRES and the stop codon at the end of P1 was examined and the EMC IRES was still there.

If put a large deletion in the EMC IRES, then saw only unprocessed P1. P2 and P3 were not made. Since P3 was not made there was no 3C protease to

process P1.

Also no virus was made, and no CPE produced by the RNA transcripts.

The importance of internal ribosomal entry generally accepted for some time now.

Involvement of Host Proteins in Viral Replication

Host Proteins are involved both in translation of viral proteins and replication of viral RNA

The La autoantigen

PV RNA is translated very poorly in cell-free lysates of rabbit reticulocytes (RRL).

If add extract from HeLa cells to RRL, then the translation of the PV RNA is stimulated.

Identified a 52k protein that binds to 5' UTR between nt's 559 and 624 (i.e. in the IRES sequence)

This protein is abundant in HeLa cell extracts, but not in RRL. Could this protein be the factor that stimulates translation of PV RNA in RRL.

Sequencing of the protein showed it to be the La autoantigen, an antigen recognized by antibodies from patients with certain autoimmune disorders such as lupus.

Showed that La protein indeed "corrects" translation of PV RNA in RRL, but has no effect on translation of non-picornavirus viral RNAs such as those of TMV and BMV

This result is consistent with the idea that La protein is important in internal initiation via IRES. Also showed that La protein helps ensure that initiation of protein synthesis begins with the proper AUG initiation codon, and that PV infection leads to redistribution of La protein from nucleus to cytoplasm.

What is the normal function of La protein? - it is a termination factor for transcription by RNA pol III, and therefore is primarily a nuclear protein.

The classical example of phage Q β : this is a small bacterial RNA virus; the viral RDRP of Q β has 4 subunits; only one is virus coded; the other three are host cell proteins involved in translation.

Translation vs RNA synthesis:

In considering translation and replication of viral RNA, an interesting problem

arises. Genome RNA is the template for both translation and for synthesis of negative-strand RNA.

Ribosomes are translating the RNA 5' to 3', while the viral RDRP copies the genome 3' to 5'. Which have the right of way, the ribosomes or the RDRP?

It was shown in an *in vitro* system that the viral RDRP is unable to replicate RNA which is being translated. However, if added cycloheximide to the system to inhibit protein synthesis, and knock off ribosomes, then RNA synthesis could proceed. Later it was shown that an RNA sequence in the 5' UTR 5' to the IRES is critical for the regulation of translation and RNA synthesis.

This sequence is contained in the cloverleaf structure of the 5' UTR (nt 1-108). This cloverleaf structure is 5' to the IRES.

When a cellular protein, poly(rC)-binding protein (PCBP), interacts with the cloverleaf structure, translation of the viral RNA is up-regulated.

However, the viral protein 3CD also interacts with the cloverleaf structure (at a different site), and results in a repression of translation and stimulation of (-) strand RNA synthesis.

PCBP binds to the B loop of the cloverleaf structure, and 3CD binds to the D loop. Thus, the binding of one cellular protein and one virus-coded protein appear to determine the balance between translation and RNA synthesis.

Work by others had shown that each molecule of viral RNA has to be translated before it is replicated. So apart from RNA which is packaged into virions, the other viral RNA molecules must be used first as a template for protein synthesis before they can be replicated.

In the uninfected cell, the PCBP's are involved in the stabilization and translational regulation of heterogenous nuclear (hn) or mRNA's.

Myristylation of VP4

Envelope proteins of viruses are often modified post-translationally to contain covalently bound fatty acids which helps anchor the protein in the viral membrane. Polio does not have an envelope. But RNA replication is membrane associated.

When experiments were done to see if any of the poliovirus proteins were acylated, found that the virion contained covalently bound myristic acid.

The fatty acid was bound to VP4 and to the precursor of VP4 i.e VP0, and specifically to the N-terminal G of VP4 or VP0 (initiating meth residue is cleaved off) the viral polypeptide.

Many viruses are now known to have a capsid protein which is myristylated, and

the myristic acid is always bound to an N-term G.

What happens if VP0 is not myristylated? An experiment was done using two recombinant vaccinia viruses (VV) as expression vectors.

VVP1 expressed the P1 polyprotein.

VVP3 expressed 3CD-pro.

When infected cells with these 2 viruses, P1 was made, 3CD^{pro} was made, and 3CD^{pro} processed P1 to VP0, VP1 and VP3. These proteins were then assembled into 75s empty procapsids.

But then took VVP1, and changed Gly-2 in P1 to Ala, so myristylation could not occur. Called this virus VVP1 myr(-).

If infected cells only with VVP1 or VVP1myr(-), P1 was made but it was not processed. This was expected. because no 3CD^{pro} present.

If infected cells with VVP1 and VVP3 observed both processing and assembly, as already noted.

But when infected with VVP1myr(-) and VVP3, did see processing to VP0, VP1 and VP3, but the proteins were unstable and did not accumulate.

Did not see 75s particles, nor 14s particles, only the 5s protomers.

Conclusion: myristylation is necessary for viral replication and specifically for assembly of 5s to 14s particles.

What is the origin of the enzyme that carries out the myristylation.

It is a cellular enzyme, and it is specific for myristylation of Gly.

The cell free synthesis of poliovirus has been accomplished

1) a cell-free extract from Hela cells was made which was able to carry out translation of PV RNA

2) It contained all the components needed for in vitro translation, ribosomes amino acids , ATP and so forth.

3) added PV RNA, and incubated for 15 hours.

All the viral proteins were made, and a low level of infectious virus (measured as pfu) was detected. Plaque formation due to the newly synthesized virus was inhibited by antiviral serum.

How was it shown that the viral RNA was actually being replicated and that added PV RNA was not simply being encapsidated by newly synthesized proteins?

1) Increasing the concentration of NTP's gave a 70-fold increase in the yield of

infectious virus..

2) PCR analysis showed presence of negative strand RNA; thus there was replication of viral RNA

Although the production of infectious virus was very inefficient in this first report, this did represent an important step in establishing an *in vitro* system for the study of viral replication.

The Poliovirus receptor

Poliovirus has a narrow host range: it infects only primates and primate cell cultures. Other species lack the receptor on the cell surface.

Some years ago, the PV receptor was identified. The gene was cloned and sequenced, and it turned out to be a new and previously unknown member of the immunoglobulin superfamily of proteins.

Expression of PVR in foreign cells and animals

Since mice and mouse cells lack the PVR receptor, they are not permissive for poliovirus.

When the PVR was expressed in mouse cells, they became susceptible to infection with PV.

It was then possible to make transgenic mice expressing the PVR. These mice were susceptible to infection with poliovirus.

Conclusions:

In mice expression of PVR is clearly a determinant of viral host range.

In humans, the situation is more complicated because virus does not replicate in all cell types that express the PVR. This could be because PVR is masked by its natural ligand.

The hope was that transgenic mice could then provide a system for testing the safety of vaccines in place of monkeys.

Further confirmation of the importance of the PVR for productive infection of cells.

A HeLa cell mutant (HeLa cells are human cells) was selected which survived infection with PV, i.e. it was resistant to the virus.

PV did not adsorb to these cells, and the cells lacked an epitope on their surface which is characteristic of the PVR.

Further studies showed that although the PVR-coding sequence was present in these cells, the amount of PVR transcript was greatly reduced.

When these cells were treated with azaserine, (a compound which interferes with

the methylation of cellular DNA), the yield of virus was increased 10^5 -fold, due to increased transcription of the PVR DNA sequence.

These results suggested that in the resistant cells, the PVR gene was hypermethylated and hence inactive.

The PV RDRP

It is now clear that all 4 types of polymerases share important structural features and catalytic mechanisms.

The model for virtually all nucleic acid polymerases is a right hand with fingers, thumb and palm domains; the catalytic site is in the palm domain.

All RDRP's share 5 sequence motifs, called A to E, going N terminal to C-terminal. More recently another motif, F, has been described which is N-terminal to the A motif.

In motif A there is a conserved D, and in motif C there is a conserved GDD sequence which is the hallmark of most RDRP's.

These D residues coordinate Mg^{++} ions which bind to the incoming rNTP molecule.

The PV RDRP was crystallized and its structure determined (except for one small region in the first report) by X-ray crystallography. This was the first structure determination of a viral RDRP.

Questions to Consider Concerning Viral RNA Synthesis

1. How to begin viral RNA synthesis at the end of the molecule and preserve the terminal sequences.

Compare RNA synthesis by a DDRP and by a RDRP.

2. How to make synthesis of a minus-strand RNA off a plus-strand viral RNA, which has a poly(A) tail, specific for viral RNA.
3. How is it determined that the plus-strand RNA is made in great excess over the minus-strand RNA?
4. What are the specific requirements for synthesis of minus-strand RNA? Are they different from the requirements for the synthesis of plus-strand RNA?
5. What is the role of host cell proteins in the synthesis of viral RNA?

Synthesis of Poliovirus RNA

The relevant cis-elements:

The cloverleaf structure in the 5' UTR

Poly(A) in the 3' UTR, and possibly 2 stem loop structures in the 3' UTR

Cis-replication element CRE (2C)

Binding of cellular proteins

Poly(A) binding protein (PABP) to 3' UTR

3CD^{pro} to stem loop D of cloverleaf structure

Poly(C) binding protein (PCBP) to stem loop B of cloverleaf structure

Circularization of viral RNA

By interaction of protein interactions: PABP with 3CD^{pro}

Formation of protein complex around CRE (2C)

By interaction with 3CD^{pro}, 3D^{pol}, and VPg

Uridylation of VPg by 3D^{pol} on AAACA template to give VPgUpU

Slideback mechanism to give 2nd or 3' U.

Synthesis of (-) strand RNA to give RF RNA which is a completely ds structure

For synthesis of (+) strand RNA

VPg is uridylylated using as template the 2 A residues at the 3' end of the (-) strand RNA

