Bluetongue virus replication, molecular and structural biology

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Summary

The icosahedral bluetongue virus (BTV) particle (~80 nm diameter) is composed of three distinct protein layers. These include the subcore shell (VP3), core-surface layer (VP7) and outer capsid layer (VP2 and VP5). The core also contains ten dsRNA genome segments and three minor proteins (VP1[Pol], VP4[CaP]and VP6[Hel]), which form transcriptase complexes. The atomic structure of the BTV core has been determined by X-ray crystallography, demonstrating how the major core proteins are assembled and interact. The VP3 subcore shell assembles at an early stage of virus morphogenesis and not only determines the internal organisation of the genome and transcriptase complexes, but also forms a scaffold for assembly of the outer protein layers. The BTV polymerase (VP1) and VP3 have many functional constraints and equivalent proteins have been identified throughout the *Reoviridae*, and even in some other families of dsRNA viruses. Variations in these highly conserved proteins can be used to identify members of different genera (e.g. by comparing the polymerase) and different virus species (serogroups) within the genus *Orbivirus* (e.g. by comparison of VP3). This has helped to identify three new genera within the *Reoviridae* and two new *Orbivirus* species. In contrast, sequences of the BTV outer capsid proteins (involved in interactions with neutralising antibodies) are much more variable (particularly VP2) and comprehensive sequence analyses for the 24 types demonstrate that they can be used to identify BTV serotype. The 21 species (158 serotypes) currently recognised within the genus *Orbivirus* are listed, along with 11 unassigned viruses.

Keywords

Bluetongue virus – Core – Molecular biology – *Orbivirus* – *Reoviridae* – Replication – Segment 1 – Segment 2 – Segment 6 – Serotype determination – Structural biology – Subcore – Viral protein 2 – Viral protein 5 – Virus structure.

Introduction: bluetongue virus classification and virion structure

The family *Reoviridae* currently contains twelve genera of multi-segmented dsRNA viruses, including pathogens of a wide range of insects, reptiles, fish, crustaceans, mammals (including humans), plants and fungi (31), many of which are of economic, veterinary or medical importance. These viruses can be distinguished and identified by a number of different characteristic features, including capsid structure, number and size distribution of genome segments, host range, serological properties, protein composition, disease symptoms and most recently by sequence analyses and comparisons of individual genome segments. Indeed, these sequencing studies and phylogenetic comparisons have helped to identify three new genera within the family *Reoviridae* and two new species of *Orbivirus* (Tables I and II) that will be included in the Eighth Report of the International Committee for the Taxonomy of Viruses (ICTV), due to be published in 2004.

The orbiviruses (which are classified as members of the genus *Orbivirus*, within the family *Reoviridae*) characteristically have a ten-segmented dsRNA genome that is packaged as one copy of each segment within an icosahedral protein capsid (~85 nm diameter). Bluetongue virus (BTV) is the prototype species of twenty-one different *Orbivirus*

Table I
Virus genera of the family *Reoviridae*
Genera of viruses with genomes composed of 10-12 segments of dsRNA

Genus	No. of genome segments	No. of species	No. of types (serotypes)	Tentative or unassigned isolates
1. *Orthoreovirus*	10	4	6	-
2. *Orbivirus*	**10**	**21**	**160**	**11**
3. *Cypovirus*	10	16	16	3
4. *Aquareovirus*	11	6	unknown	5
5. *Rotavirus*	11	5	>23	2
6. *Coltivirus*	12	2	5	1
7. *Seadornavirus*	12	3	7	15
8. *Fijivirus*	10	8	8	-
9. *Phytoreovirus*	12	3	3	1
10. *Oryzavirus*	10	2	4	-
11. *Mycoreovirus*	11 or 12	3	3	-
12. *Idnoreovirus**	10	5	6	1

* the creation of the three new genera *Seadornavirus*, *Mycoreovirus* and *Idnoreovirus* has been approved by the International Committee for the Taxonomy of Viruses. The name *Idnoreovirus* is derived from '**I**nsect **d**erived **n**on-**o**ccluded **reovirus**'

Table II
The *Orbivirus* species

***Orbivirus* species currently recognised**	**No. of serotypes/strains**	**Tentative species/ unassigned viruses**
1. African horse sickness virus (AHSV)	9 serotypes	
2. Bluetongue virus (BTV)	24 serotypes	Andasibe virus (ANDV)
3. Changuinola virus (CGLV)	12 serotypes	Ife virus (IFEV)
4. Chenuda virus (CNUV)	7 serotypes	Itupiranga virus (ITUV)
5. Chobar Gorge virus (CGV)	2 serotypes	Japanaut virus (JAPV)
6. Corriparta virus (CORV)	6 serotypes/strains[a]	Kammavanpettai virus (KMPV)
7. Epizootic haemorrhagic disease virus (EHDV)	10 serotypes/strains[a]	Lake Clarendon virus (LCV)
8. Equine encephalosis virus (EEV)	7 serotypes	Matucare virus (MATV)
9. Eubenangee virus (EUBV)	4 serotypes	Tembe virus (TMEV)
10. Ieri virus (IERIV)	3 serotypes	Codajas virus (COV)
11. Great Island virus (GIV)	36 serotypes/strains[a]	Tracambe virus (TRV)
12. Lebombo virus (LEBV)	1 serotypes	Yunnan orbivirus (YOV)
13. Orungo virus (ORUV)	4 serotypes	
14. Palyam virus (PALV)	13 serotypes/strains[a]	
15. Peruvian horse sickness virus (PHSV)[b]	1 serotype	
16. St Croix River virus (SCRV)[b]	1 serotype	
17. Umatilla virus (UMAV)	4 serotypes	
18. Wad Medani virus (WMV)	2 serotypes	
19. Wallal virus (WALV)	3 serotypes/strains[a]	
20. Warrego virus (WARV)	3 serotypes/strains[a]	
21. Wongorr virus (WGRV)	8 serotypes/strains[a]	
Total 21 virus species	160 serotypes/strains[a]	11 unassigned viruses

a) in some species the serological relationships between strains has not been fully determined

b) two new species of *Orbivirus* (SCRV and PHSV) have recently been recognised by the International Committee for the Taxonomy of Viruses, based primarily on genome segment sequence analyses and comparison (3, 33); Ndelle virus (previously classified as an *Orbivirus*) was also reclassified as an *Orthoreovirus* on this basis (2)

species now recognised by the ICTV. It has a capsid composed of three distinct protein layers (Figs 1 and 2): the subcore, composed of VP3 (T2); the core-surface layer, composed of VP7 (T13); and the outer capsid layer, composed of VP2 and VP5.

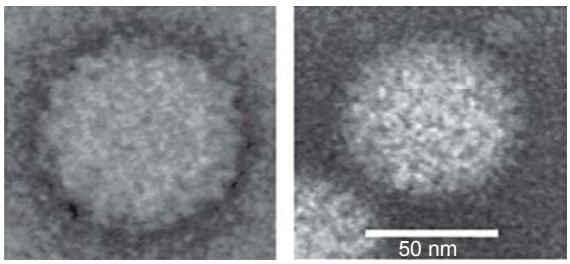

Figure 1
Electron micrographs of negatively stained virus and core particles of BTV-1, purified
Mertens *et al.* (28)

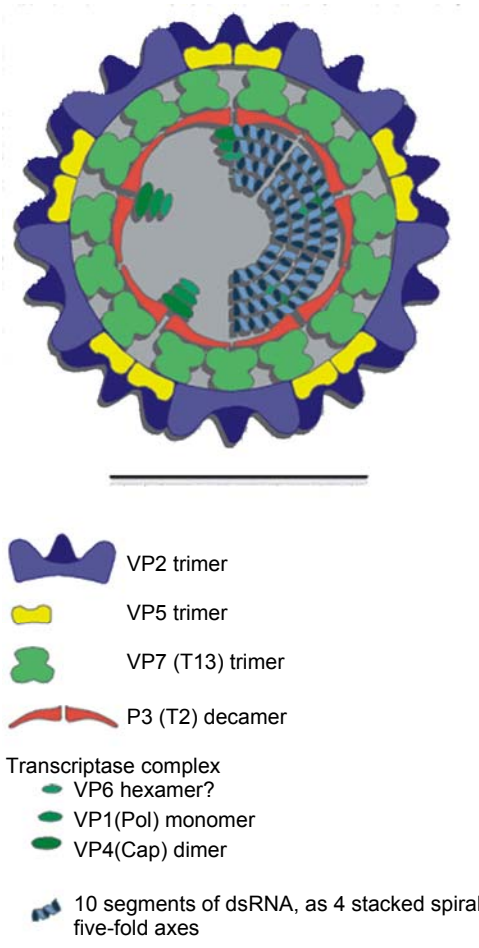

Figure 2
Schematic diagram illustrating the structure of the bluetongue virus particle derived from biochemical, X-ray crystallography and cryo-electron microscopy

The earlier development of effective purification methods for BTV virus particles and cores (Figs 1 and 3) (28) not only provided material for electrophoretic analyses and biochemical studies of viral proteins/RNAs. It also provided homogeneous particles in sufficient quantity and purity for crystallisation (Fig. 3) and X-ray diffraction studies of the core particle (5) leading to the resolution of its atomic structure (6, 10, 13). It has not been possible to crystallise intact BTV virus particles and consequently it has not yet been possible to derive a high-resolution atomic structure for components of the outer capsid shell. However, the BTV outer capsid structure has previously been determined by cryo-electron microscopy (EM) (15, 16). These studies demonstrated that it is composed of 180 copies of the Mr 111 × 10^3, 'sail-shaped' VP2 protein, arranged as trimeric 'triskellion' structures, together with 360 copies of an inter-dispersed and underlying VP5 protein (Mr 59 × 10^3), which also appears to be arranged as 120 trimers. Figure 2 shows a schematic representation of the BTV particle structure derived from these X-ray crystallography and EM structural studies. Additional biochemical data concerning the individual BTV proteins are available on the dsRNA virus page on the Institute for Animal Health website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/Orbivirus.htm).

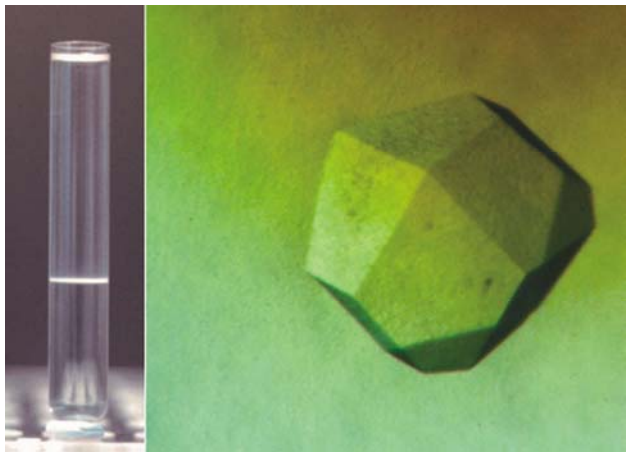

Figure 3
A self forming CsCl gradient used to purify core particles of BTV-1 and a typical crystal of native BTV-10 core particles
The image on the left shows a self-forming CsCl gradient used to purify core particles of BTV-1 (28)
The presence of only a single band that contains ~20 mg of core particles demonstrates the homogeneity and purity of the core preparation
The image on the right shows a typical crystal of native BTV-10 core particles, approximately 0.5 mm in diameter (5)
>1 000 of these crystals were used in studies to determine the atomic structure of the BTV core (13)

The bluetongue virus outer-capsid proteins and determination of serotype

The structure of the outer capsid layers of the different species and genera of viruses within the

family *Reoviridae* are highly variable (31), reflecting their responsibility for virus transmission between individuals of widely different host species, and their role in mediating cell attachment and penetration of different cell types. In many cases, it is impossible to identify exactly comparable outer capsid proteins from the different genera. In mammalian hosts, these outer surface proteins also interact with components of the immune system of the hosts, inducing neutralising antibodies. Consequently they are subject to antibody selective pressure, leading to higher levels of sequence variation even within a single virus genus or species.

Indeed, the components of the BTV outer capsid, proteins VP2 and VP5, are the most variable of the viral proteins. VP2, in particular, contains neutralising epitopes and by controlling the specificity of virus particle interactions with neutralising antibodies, determines the identity of the 24 BTV serotypes that are currently recognised using serum neutralisation (SN) assays. Sequence analyses of genome segment 2 (23, 24, 25) and segment 6 (37) from representative isolates of all 24 BTV serotypes have recently been completed. Phylogenetic comparisons have demonstrated that variations in the nucleotide sequences of segment 2 and 6 and in the amino acid sequences of VP2 and VP5 show a high correlation with virus type as determined by SN assays (23, 24, 25, 37). This is particularly true for BTV genome segment 2, where oligonucleotide primers designed for reverse transcriptase-polymerase chain reaction (RT-PCR) assays and sequencing studies can be used to rapidly and reliably identify virus serotype (23, 24, 25).

Bluetongue virus cell entry and initiation of virus replication

A schematic representation of the BTV replication cycle is shown in Figure 4 (27). Infecting BTV particles are taken up via an endosomal route (19). The reduction of pH within the early endosome is thought to release outer capsid components from the virus core, which is then released into the host cell cytoplasm. Individually expressed VP5 is toxic, causing cell fusion (14) and is consequently thought to play a role in penetration of the endosomal membrane and the release mechanism. However, the BTV core particle contains no VP5, but it is also infectious in its own right for both *Culicoides* and some mammalian cell systems (30). Antibodies to the outer core protein VP7 will bind to and neutralise core particles but not fully intact virus (18). This suggests that although VP7 (which is not exposed on the surface of fully intact virions) (18) can mediate both cell attachment and penetration of the BTV core, it is not involved in cell attachment of the intact virion. The BTV core can bind to the cell surface via the interaction of VP7 with glycosaminoglycans, although other receptors may also be required for cell entry (18). Core particle infectivity, unlike that of the intact virus, is also independent of the pH reduction in the endosome (18) and is unaffected by treatment of cells with either ammonium sulphate or Concanamycin A (a drug

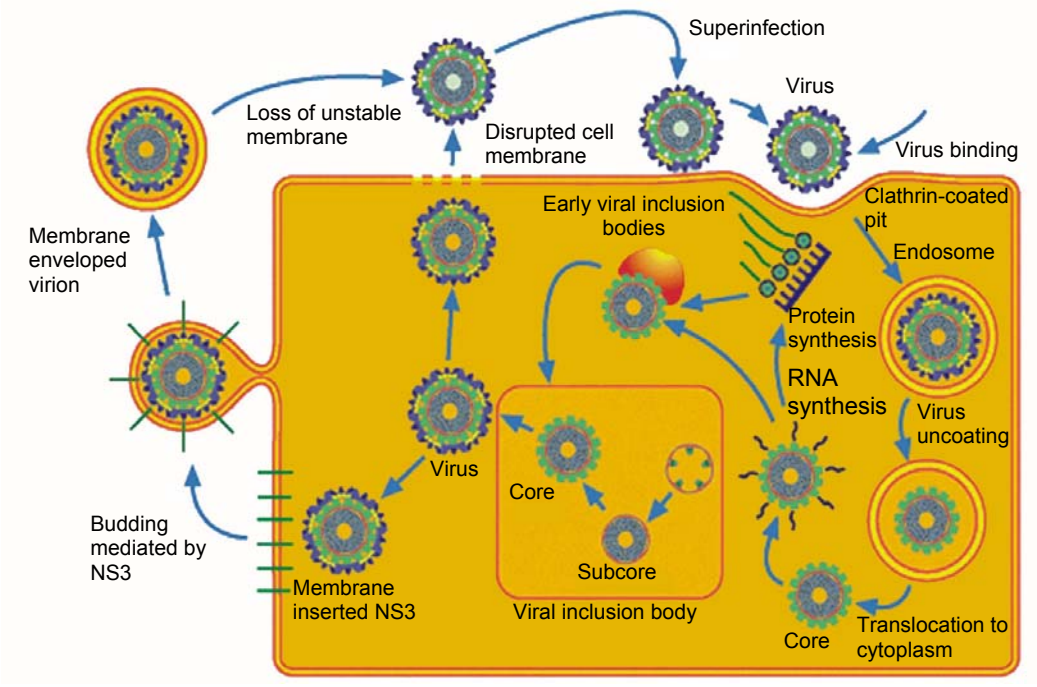

Figure 4
Schematic diagram representing the lytic replication cycle of bluetongue virus (27)

that specifically blocks the vacuolar ATPase, thereby raising the pH of the endosome). It appears likely that this reflects the absence of an outer capsid layer that must be removed in order to activate the core-associated RNA-dependent RNA polymerase. It also suggests that release of the outer capsid proteins or exposure of VP7 may be essential for transport of the core into the cell cytoplasm from the endosome. However, it is also possible that the BTV core enters cells via a different route.

Virus replication and transcription

In order to infect and successfully replicate in their target host species, the dsRNA viruses have to overcome a number of specific biochemical problems. dsRNA molecules are ineffective as mRNA for translation and cannot themselves function as templates for host cell transcriptases. BTV must therefore provide its own transcription and capping enzymes and carry them into the host cell at the initiation of infection, in order to synthesise the mRNAs required for synthesis of viral proteins. However, many host cells also contain antiviral defence mechanisms (20) including induction of apoptosis, interferon production, modification of host-cell translation mechanisms and even RNA silencing (8, 9). Many of these mechanisms recognise and would be activated by naked dsRNA within the host cell cytoplasm (20). Indeed, the ubiquitous nature of these defences in many different animals and plants suggests that the dsRNA viruses are themselves an ancient lineage that has diversified, along with their host species, from common ancestors.

In order to avoid exposure to the host cell cytoplasm and activation of host defences, many of the dsRNA viruses (including the orbiviruses) retain their genomes, and mRNA synthesising enzymes, within stable closed-protein capsids. These 'nano' transcription machines form the basic infectious unit of the virus and must be delivered intact into the host cell cytoplasm in order to initiate replication. The removal of viral proteins VP2 and VP5 during cell entry activates the transcriptase functions of the core particle, allowing it to synthesise and cap full length mRNA copies of the ten genome segments, while they are still packaged within the core itself. Some further disassembly of cores may occur (17), although this is not thought to be a functional part of the replication process and the majority of core particles appear to remain intact throughout the replication cycle (7).

The major protein components of the bluetongue virus core structure

The structures of the core of BTV serotypes 1 (BTV-1) and 10 (BTV-10) (containing ~1 000 protein molecules) have been determined to a resolution of 3.5 Å and 6.5 Å respectively, the latter using crystals with unit cell parameters in excess of 1 000 Å (10, 13). These structures have revealed the organisation of the protein bilayer that makes up the icosahedral capsid of the BTV core.

The innermost complete capsid shell of the BTV core (the subcore) is composed of 120 copies of the viral protein VP3 (901 amino acids, 103 kD), arranged with T=2 icosahedral pseudo-symmetry (Fig. 5). This requires VP3 to occupy two different conformations, identified as A and B, in order to form a complete capsid shell. The VP3 A and B molecules are chemically identical but undergo a conformational shift between their internal 'apical', 'carapace' and 'dimerisation' domains, allowing them to interact and close the surface of the icosahedron.

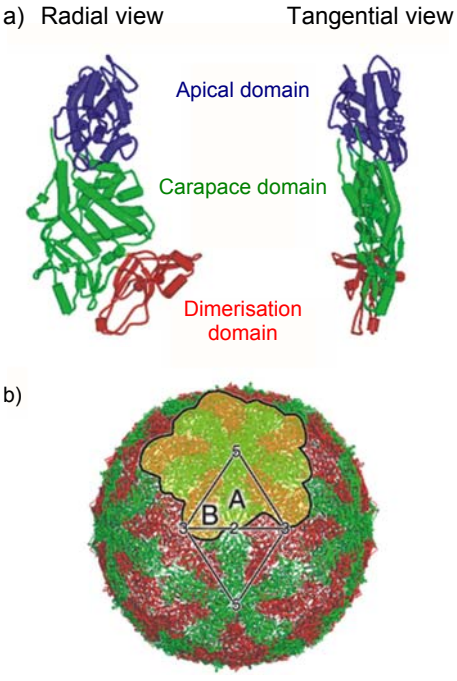

Figure 5
a) Diagram showing the domain structure of VP3 (T2)
The radial view shows the molecule as if it were in the subcore when viewed from outside the particle looking towards the centre
The tangential view shows how thin the molecule, and consequently the subcore, shell is compared to the volume it encloses

b) The bluetongue virus subcore, made up of 120 copies of VP3 (T2)
The ten conformationally distinct A and B molecules making up a decamer at the uppermost five-fold axis are outlined
The icosahedral five-fold, three-fold and two-fold symmetry axes have been marked
Grimes *et al.* (13)

The subcore can be considered as an assembly of twelve VP3 decamers, each containing five copies of the triangular VP3-A molecules around the five-fold apex of the icosahedron, leaving a small central pore. These are interspersed with five VP3-B molecules that are slightly more distant from the five-fold axis, creating a dish-shaped decamer. Each of the 12 decamers has a zigzag outer edge that 'zips' together with its neighbours to form the intact icosahedral shell.

The BTV subcore layer can self-assemble when VP3 is synthesised separately from the other viral proteins (for example as expressed by recombinant baculovirus in insect cells) (P. Roy, personal communication). Subcore particles of some orbiviruses are also stable in the absence of the outer capsid layers (Fig. 6). Structure-based modification of the VP3 molecule to remove the dimerisation domain responsible for inter-decamer contacts still allows decamer formation but prevents assembly of the intact subcore shell (P. Roy, personal communication) supporting a model for subcore assembly from 12 interacting decamers.

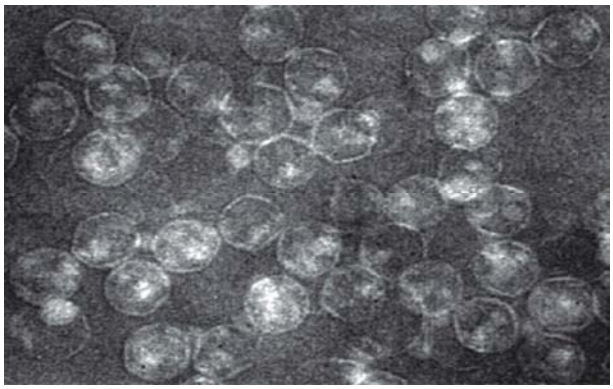

Figure 6
Subcore particles of equine encephalosis virus (EEV) a member of a distinct *Orbivirus* species purified using modifications of the method described for BTV cores
These particles were stable in 0.1M Tris/HCl pH 8.0
Condensed internal material (possibly RNA) can be seen in many particles
Burroughs *et al.* (5)

The architecture of the BTV subcore shell and the overall shape of the subcore shell protein (VP3 of BTV) show remarkable similarities to the innermost capsid shell and inner capsid protein of many other dsRNA viruses. This suggests that these dsRNA viruses evolved from a common ancestor that originally developed a simple and elegant mechanism for the assembly of an inner capsid shell. This now represents an important and characteristic step in the virion assembly pathway. The amino acid sequence of VP3 must determine not only the fold and overall structure of the protein, but also it represents the 'information' that allows the subcore shell to self-assemble. Once completed, the BTV subcore can act as a scaffold for the attachment of VP7 and subsequently the outer capsid proteins VP2 and VP5, thereby influencing the structure of whole capsid. The VP3 (T2) layer also dictates the internal organisation of the viral genome and the components of the transcriptase complexes (VP1, VP4 and VP6) that are attached, or interact with it internally. This protein therefore carries an enormous functional load and as a result it is highly conserved within the different genera of the *Reoviridae*. Indeed functionally and structurally similar subcore shell proteins can be identified in many other dsRNA viruses. This allows them to be used in phylogenetic analyses of the ancestral relationships of these viruses, as has previously been described for BTV (11, 12). By comparing the sequences of genome segment 3 and the VP3 protein, it is possible to distinguish different species within the genus *Orbivirus* and such comparisons provided important evidence to support the classification of St Croix River virus as a new species of *Orbivirus* (3).

The outer surface of the BTV core contains 780 copies of the VP7, arranged (as 260 trimers) with T = 13*l* icosahedral symmetry. Individual trimers can occupy five different positions in the core surface layer, which are identified with increasing distance from the five-fold axis, starting with P through Q, R and S, to T at the three-fold axis (Fig. 7).

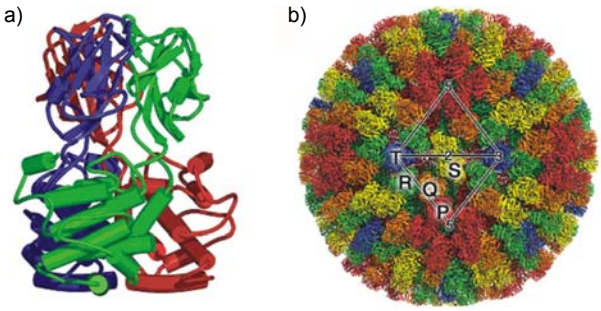

Figure 7
a) Diagram of the structure of the VP7 trimer
The trimer sits on the exterior surface of the subcore, making contact through its base as shown here
Contacts between VP7 trimers are mediated through the sides of the lower half of the molecule

b) Diagram showing the core surface structure
The icosahedral five-fold, three-fold and two-fold axes are labelled and the icosahedrally independent trimers P, Q, R, S and T are identified (13)

These VP7 molecules are layered onto the scaffolding of the intact VP3 (T2) subcore shell. As a result of mismatched symmetry between these layers of protein, the base of the VP7 monomers interact

with the surface of the underlying subcore VP3 (T2) molecules in thirteen different orientations. These interactions curve the layer of VP7 trimers around the subcore particle surface, as a series of six component, ring-shaped capsomers, finishing with rings of five VP7 trimers around the five-fold axes. The significance of VP3-VP7 interactions in forming the core surface layer is suggested by VP7 of African horse sickness viruses (AHSV) (members of another closely related *Orbivirus* species), which form crystals of VP7 within the cell cytoplasm. In the absence of any interactions with VP3 (T2), the VP7 trimers form into large flat hexagonal arrays of exclusively six-membered rings (4). Only at the three-fold icosahedral axes of the BTV core do the symmetries of the two protein layers of the core particle coincide. At this point, the interactions between the VP7 (T13) layer and VP3 (T2) layer are also the most extensive, suggesting that the T trimer is added first and the other trimers are added sequentially towards the five-fold axes.

RNA packaging

Electron density maps of the core of both BTV-1 and BTV-10 (10, 13) have revealed layers of density within the central space of the subcore that cannot be modelled as viral proteins (Fig. 8). These layers are made up of multiple strands, which in many places have a helical structure that is not only very similar for the two virus serotypes but are also consistent with layers of the packaged genomic dsRNA (Fig. 9).

From a detailed knowledge of the volume and internal contents of the BTV core (13), it was possible to calculate the concentration of the dsRNA within the central cavity of the BTV particle at ~410 mg/ml.

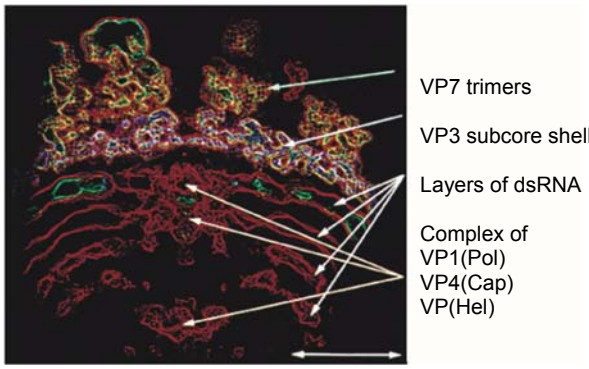

Figure 8
A slice through the electron density in the bluetongue virus core
The strong density of the VP7 and VP3 molecules of the capsid layer is visible towards the top of the figure
Within the capsid, up to four layers of density are visible away from the five-fold axis
These layers are of thickness and spacing consistent with their being due to dsRNA (10)
At the five-fold axis is a section of unlayered density that is thought to represent the protein components of the transcriptase complex

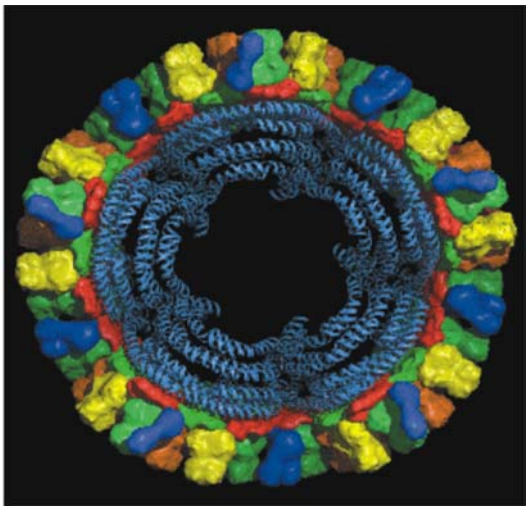

Figure 9
Cross section through the bluetongue virus core structure, as determined by X-ray crystallography cores of BTV-1 and BTV-10
Four layers of dsRNA are shown, as stacks of spirally arranged helices, at each of the five-fold axes
The genomic RNA is surrounded by the outer core (VP7 [T13]) and subcore shells (VP3 [T2])

The properties of concentrated solutions of dsDNA are relatively well characterised. It is established that it forms liquid crystalline arrays at high concentration, with the phase and helix-helix packing distance being a simple function of concentration (22) (although less is known about the properties of dsRNA). At concentrations of ~400 mg/ml, the liquid crystalline packing arrangement for DNA has been shown to be columnar hexagonal, with an inter-helix packing distance of approximately 30 Å and it appears likely that the behaviour of dsRNA would be similar at high concentrations. This ordering of the RNA may be essential if it is to function effectively as a template for the virion-associated transcriptase activities, without becoming tangled and jamming the mechanism. One potentially relevant property of this packing structure is that in the presence of suitable counter ions the nucleic acid chains would glide over each other with very little friction (22). The phosphate backbone of the dsRNA carries a negative charge that would presumably be neutralised by counter ions within the particle. However, a scanning proton microprobe, used to detect the presence of metal ions within the crystals of BTV core particles (10), failed to detect magnesium. Although both calcium and zinc were present, they are only detected at approximately one hundredth of the level of phosphorous. This would be insufficient to neutralise the charge on the phosphate backbone of the RNA and suggests that

an organic cation such as spermidine may be present, although this has not yet been confirmed.

The model proposed for the packing of the BTV genome (derived from X-ray crystallography studies) (10) represents ~80% of the 19 219 bp (a total length ~6 μm). This model implies that there is a particular organisation of the dsRNA strands within the core. Such ordering appears to be at least partially imposed by chemically featureless grooves that form tracks for the RNA on the inside of the VP3 (T2) layer. Specific RNA/protein interactions are evident at only two points in the icosahedral asymmetric unit (13) and there are very few basic residues on the inner surface of VP3 (T2). This paucity of specific interactions may also facilitate the movement of RNA within the core (for example during transcription).

There are striking similarities in the interactions that the structurally distinct A and B copies of VP3 (T2) make with the RNA (although there are also some differences reflecting the specific conformational changes between the two protein subunits). Since the two molecules lie at different radial distances from the five-fold axes, the protein-RNA interactions generate a spiral structure that is observed in the RNA. The model proposed by Gouet *et al.* (10) shows each dsRNA strand in the outer shell of packaged RNA, leaving the transcriptase complex (TC) situated on the inner surface at the five-fold axis of the subcore shell, then spiralling around it (10). At a certain diameter away from the five-fold axis, the RNA effectively fills the outer layer and interacts with a neighbouring genome segment. This is thought likely to redirect the RNA strands inward to lay down a second discrete layer, spiralling back toward the TC at the five-fold axis. Further switching would lay down the third and fourth layers (Fig. 9). Although no electron density is seen connecting these layers, icosahedral averaging would render a single link invisible.

The BTV genome segments have very different lengths (from 822 to 3 954 bp) and there is little free space within the particle. Some of the longer dsRNA molecules must therefore trespass into the volume of neighbouring segments (as well as into the volume around the two five-fold axes that are empty of genome). This suggests that simple steric clashes with neighbouring segments might be one of the most important factors limiting the lateral expansion of the spiral (particularly in the inner layer of the RNA), although it is possible that layer switching in the outer RNA layer might also be facilitated by interaction with a flexible loop of VP3-B, close to the icosahedral three-fold axis.

The minor protein components of the bluetongue virus core

The core particle represents a biochemically active compartment. It contains the ten dsRNA genome segments, as well as enzymes needed to synthesise cap and methylate ssRNA copies of each segment. These mRNAs are released into the cell cytoplasm, where they are either translated into viral proteins, initiating the molecular events of virus replication and morphogenesis, or are processed by the enzyme components of nascent core particles and packaged as part of new viral genome segments within progeny virus particles (Fig. 4).

The BTV core contains a small number (10 to 12 copies) of transcriptase complexes (TC) (39) which are activated by removal of the outer capsid proteins. The TCs are composed of three minor structural proteins: the polymerase VP1(Pol) (36, 40) the capping enzyme VP4(Cap) (29, 32) and the helicase VP6(Hel) (38). The polymerase, which requires Mg^{2+}, transcribes positive (+ve) sense ssRNA copies from the dsRNA genome segments (Fig. 4). At a later stage of virus replication it also uses these mRNAs as a template for the production of new dsRNA genomic material, which are packaged within the progeny virus subcore. During RNA synthesis the polymerase uses NTPs as substrates and produces pyrophosphate (PP_i) as a by-product. The newly synthesised mRNA strands are capped by VP4(Cap), which has nucleotide phosphohydrolase, guanylyltransferase and two transmethylase activities that are required for synthesis of 'Cap 1' structures (21, 26, 29, 32, 34).

The nucleotide phosphohydrolase activity removes the gamma phosphate from the 5′ G residue at the end of the +ve sense RNA strand, releasing monophosphate (P_i) as a by-product. The guanylyltransferase activity then takes GDP or GTP substrates and adds GMP to the 5′ end-forming GpppG as a 5′ to 5′ linked structure, releasing P_i or PP_i as by-products. Finally the methyltransferase activities use S-adenosyl-L-methionine (AdoMet) as substrate, adding two methyl groups to the Cap structure, releasing S-adenosyl-L-homocysteine (AdoHCy) as a further by-product. VP6(Hel) has helicase and ATPase activities, using the energy released by the hydrolysis of ATP to ADP and P_i to separate dsRNA into its component strands.

In addition to the RNA of the viral genome, a distinct but unlayered region of electron density was detected within the central space, below the internal five-fold axes of the icosahedral BTV subcore, (Fig. 8). This is thought to represent the protein components of the TCs (10). These complexes

appear to be attached to the inner VP3 (T2) surface immediately below a pore at the five-fold axis. By analogy with the cypoviruses (41) and complying with the model for packing of BTV RNA described above (10), each TC is thought to be closely associated with a single genome segment (29, 40). These complexes composed of the minor core proteins, transcribe the ten genome segments, producing exactly full length mRNA copies, which are extruded from the core surface.

During transcription, each dsRNA genome segment must move through the active site of the polymerase enzyme. The fully conservative nature of BTV transcriptase imposes certain topological requirements on the process. The two strands of the parental dsRNA segments must initially be unwound prior to transcription to allow the –ve sense template strands to enter the polymerase active site. However, the resulting parental-daughter strand duplexes must also be separated, so that the nascent RNA chains can be exported from the core particle through the pores in the VP3 (T2) subcore shell at the five-fold axes (10, 13) and the parental strands of the dsRNA duplex re-annealed. It appears likely that the helicase activity of VP6(Hel) (38), is involved in one or both of these processes.

The BTV core faces several logistical problems during transcription. It must provide entry routes and mechanisms to continuously feed the vital NTP and AdoMet substrates to the internal enzyme complexes, maintain appropriate levels of certain metal ions and allow the reaction by-products to escape. It must also simultaneously propel the ten nascent mRNA molecules into the infected cell cytoplasm so that they can be translated or packaged within the next generation of virus particles. Further X-ray crystallography studies of the biochemically active core particles within intact crystals have identified sites of binding as well as entry and exit routes for the substrates and products of the core-associated enzymes. These are thought to be situated as twelve transcriptase complexes inside the subcore shell just below the pores that allow exit of the viral mRNAs (6).

The polymerase (VP1 of BTV) serves a central and vital role in the replication of dsRNA viruses. It is responsible both for the synthesis of the viral mRNAs as templates for translation of viral proteins, as well as being responsible for minus strand synthesis, usually within the nascent progeny virus particles. Consequently, the polymerase protein and the RNA segment from which it is translated are amongst the most highly conserved of the viral proteins/RNAs and can be identified across the whole of the *Reoviridae* and even in other families of dsRNA viruses. Comparisons of nucleotide and amino acid sequences of the polymerase gene/protein can therefore be used to distinguish and identify the members of different genera within the family *Reoviridae* (Fig. 10). Indeed, such a comparison formed a central component of the evidence that the seadornaviruses are distinct from members of the genus *Coltivirus* (1), leading to their classification within a distinct genus by the ICTV (Fig. 10).

Virus assembly and particle release

BTV subcore and core particles are assembled within large granular matrices, or viral inclusion bodies (VIB), within the cytoplasm of infected cells. The manner in which the genome segments and the three viral enzymes are selected for packaging within the subcore is still unresolved and represents one of the challenges that faces molecular virology. The outer capsid proteins appear to be added to the progeny core as it is released from the VIB surface.

BTV particles are released from the infected mammalian cell either by budding, or by direct cell membrane penetration, which appears to damage the cell, culminating in cell lysis. Previous expression studies have demonstrated that BTV protein NS3 (encoded by genome segment 10) can mediate virus release from insect cells. Since BTV infection does not cause cell lysis in *Culicoides* cells, NS3 may be necessary, both for cell exit and for virus spread from the initial site of infection in the insect mid-gut to the salivary glands of the insect. It may therefore control the ability of the insect to transmit the virus. Indeed studies of reassortant virus strains of AHSV, containing variants of NS3, showed considerable differences in their abilities to cause a systemic infection in adult *Culicoides* when administered via an oral route (35). This suggests that variations in NS3 sequence may influence the efficiency of virus transmission in different insect vector populations.

Conclusion

The structure and structural proteins of the multi-segmented dsRNA viruses within the family *Reoviridae*, appear to show a gradient of variability. The most conserved of these proteins are situated within the central compartment of the virion (the subcore), reflecting similar biochemical replication strategies and processes (e.g. the polymerase) or similar particle assembly mechanisms (e.g. the 'T2' subcore shell protein). The conservation evident in these proteins/segments allows them to be used to identify distantly related viruses, from different genera within the *Reoviridae* (by comparison of the

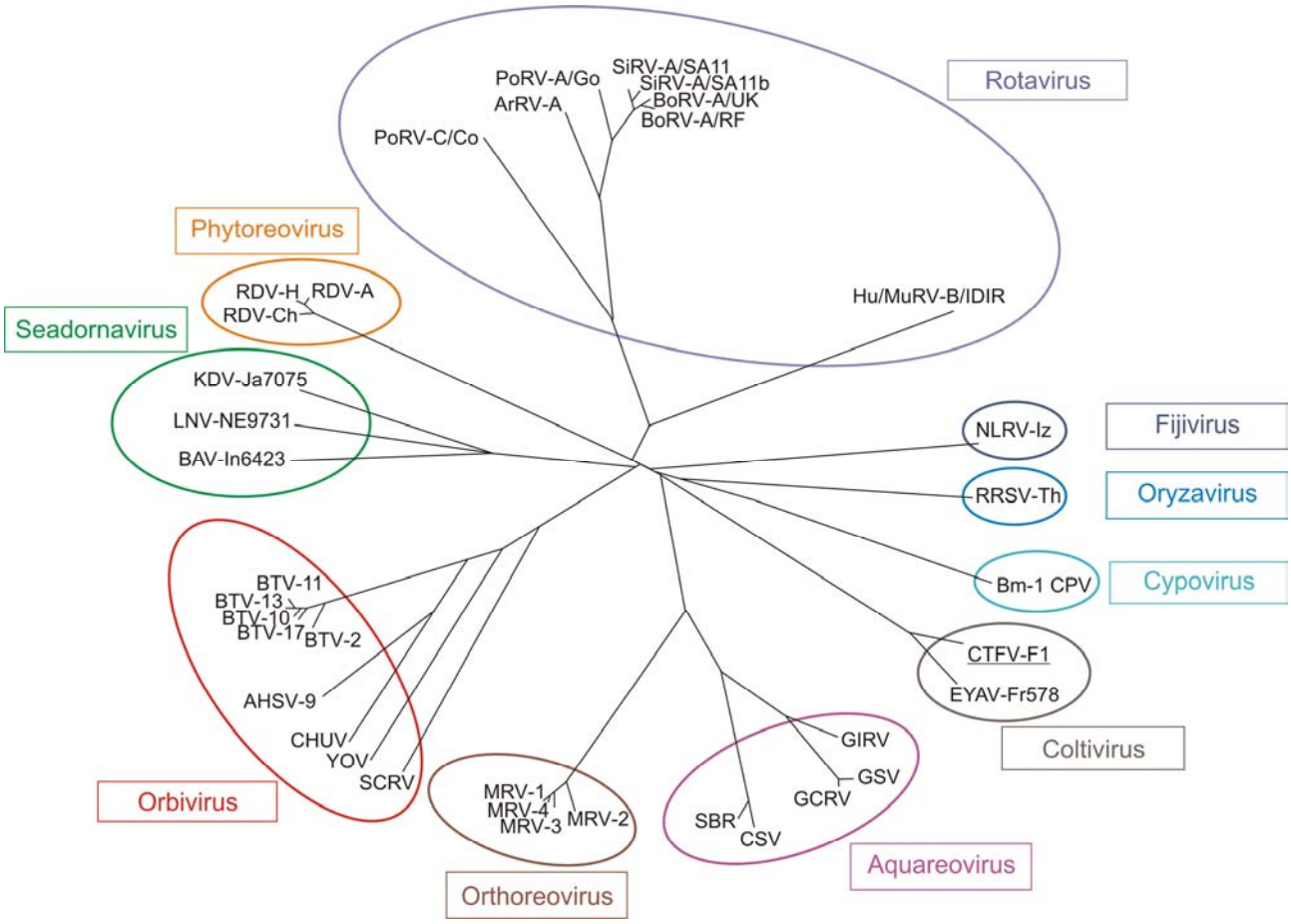

Figure 10
Neighbour joining tree for sequences of putative RNA-dependent RNA polymerases of representative viruses from genera of the family *Reoviridae*
Seadornavirus species (12 segments), *Banna virus*: isolate BAV-In6423 [AF133430], *Kadipiro virus*: isolate KDV-Ja7075 [AF133429]
Coltivirus species (12 segments), *Colorado tick fever virus*, isolate CTFV-FI [AF134529]
Orthoreovirus species (10 segments), *Mammalian orthoreovirus*, serotype-1 (MRV-1) [M24734], serotype-2 (MRV-2) [M31057], serotype-3 (MRV-3) [M31058], *Ndelle virus* (NDEV) [AF368033]
Orbivirus species (10 segments), *African horse sickness virus*, serotype-9 (AHSV-9) [U94887], *Bluetongue virus*, serotype-2 (BTV-2) [L20508], serotype-10 (BTV-10) [X12819], serotype-11 (BTV-11) [L20445], serotype-13 (BTV-13) [L20446], serotype-17 (BTV-17) [L20447], *Palyam virus*, isolate CHUV [Baa76549]
Rotavirus species (11 segments), *Rotavirus A* (RV-A), strain BoRV-A/RF [J04346], strain BoRV-A/UK [X55444], strain SiRV-A/SA11b [X16830], strain SiRV-A/SA11 [AF015955], strain PoRV-A/Go [M32805], strain AvRV-A [Baa24146], *Rotavirus B*, strain Hu/MuRV-B/IDIR [M97203], *Rotavirus C*, strain PoRV-C/Co [M74216]
Fijivirus species (10 segments), *Nilaparvata lugens reovirus*, strain NLRV-Iz [D49693]
Phytoreovirus species (12 segments), *Rice dwarf virus*, isolate RDV-Ch [U73201], isolate RDV-H [D10222], isolate RDV-A [D90198]
Oryzavirus species (10 segments), *Rice ragged stunt virus*, strain RRSV-Th [U66714]
Cypovirus species (10 segments), *Bombyx mori cytoplasmic polyhedrosis virus*-1 strain Bm-1 CPV [AF323781]
It was found that all member viruses of a single genus have AA identity over 30% (Fig. 3a), the only exception is the *Rotavirus B*, which is only 22% identical to other rotaviruses
Between member-viruses of the genera *Aquareovirus* and *Orthoreovirus*, the AA identity ranged between 40% and 42%

polymerase), or from different species for example within the genus *Orbivirus* (by comparison of VP3). In contrast, the structure of the outer capsid layers and the sequence of outer capsid proteins/genes are more variable, reflecting the different host species targeted by each virus and differences in their transmission or infection strategies. The outer capsid proteins may also interact with the immune system of the hosts, becoming subject to antibody selective pressure leading to greater variability even within a single virus species. As a result, variability in these outer capsid proteins can often be used to identify even closely related viruses. The completion of representative sequences of genome segment 2 and 6 (encoding outer capsid proteins VP2 and VP5) for each the 24 BTV serotypes, provides a database for the rapid identification of BTV serotypes and even the identification of vaccine and field strains from recent outbreaks in Europe (23, 24, 25, 37).

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Information concerning the individual proteins and RNAs of double stranded RNA viruses from several different genera is available on the dsRNA virus page on the Institute for Animal Health website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/).