

THE PESTIVIRUSES: WHERE DO THEY BELONG ?

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Résumé

LES PESTIVIRUS. — Dans cet exposé introductif, l'auteur cherche à définir la place des pestivirus parmi les virus à ARN et décrit brièvement les principales caractéristiques de ce genre de virus.

Until recently, the mainstream of RNA virology contained the naked viruses (picorna-, reoviruses) and the negative-stranded representatives of enveloped viruses (rhabdo-, orthomyxoviruses). In the seventies togaviruses became popular, and a host of information accumulated on especially Sindbis and Semliki forest virus. When we coined the collective term «non-arthropod-borne togaviruses» (Horzinek 1973a,b) it was to label a number of serologically unrelated but structurally similar viruses and to distinguish them from those agents which today are clustered in the alphavirus genus of the togaviridae and in the flaviviridae family (table 1). The nonarbo togaviruses were and still are a mixed bag which contains — amongst others — the pestiviruses, the topic of today's meeting.

In the meantime, positive-stranded enveloped RNA viruses have become quite in vogue. Mouse hepatitis virus and infectious bronchitis virus of chickens have been extensively studied since these coronaviruses have a unique mechanism of replication, which involves the formation of a nested set of messenger RNAs with 3' co-terminal ends and unique sequences extending in the 5' direction (Spaan *et al* 1981, Stern and Kennedy 1980). UV-transcription mapping has shown that the subgenomic RNAs are not processed or spliced from a common precursor. Our data are consistent with the model of an independent initiation of transcription on a genome-sized negative stranded template or on smaller templates (Jacobs *et al* 1981).

Another group of enveloped positive stranded RNA viruses for which the family status has been proposed, are the toroviruses (Horzinek and Weiss 1984). These viruses have been discovered independently in Breda/Iowa (Woode *et al* 1982) and Berne/Switzerland (Weiss *et al* 1983) and are unique in many respects, eg their polypeptide composition (Horzinek *et al* 1984, 1985, 1986, Koopmans *et al* 1986), detergent stability (Weiss

and Horzinek 1986), requirement of a nuclear phase during replication (Horzinek *et al* 1984) and morphopoiesis (Weiss and Horzinek 1986). They possess a very pleomorphic appearance caused by the varying conformation of a tubular nucleocapsid which eventually may confer the shape of a biconcave disk to the virion. Like in coronaviruses, multiple subgenomic messengers are encountered in infected cells (Horzinek, unpublished results).

Both corona- and toroviruses possess nucleocapsids of helical symmetry. This structural detail, which is still of overruling taxonomic significance, clearly distinguishes them from the togaviridae which were established to accommodate enveloped viruses with a capsid of non-helical, probably icosahedral symmetry (Andrews 1970). Also the flaviviridae, formerly a genus within the togavirus family, show this capsid architecture but they have been assigned an independent status due to their different replication strategy. At the time of the appearance of the first monograph on nonarbo togaviruses (Horzinek 1981) the following definition has been given: togavirions are spherical, 40-70 nm in diameter, and consist of an isometric, probably icosahedral, nucleocapsid, tightly surrounded by a lipoprotein envelope; the viral membrane contains host cell lipid and one to three virus-specified polypeptides, one or more of which are glycosylated. The nucleocapsid, constructed from one nonglycosylated polypeptide, contains a single colinear molecule of single-stranded RNA (molecular weight about 4×10^6 daltons), which is infectious when extracted and assayed under appropriate conditions. Togaviruses multiply in the cytoplasm and mature by budding.

It is obvious that structural details as given in this definition are not longer considered sufficient for classification. Of great taxonomic significance (due to evolutionary implications) is the replication mechanisms of a virus. With other words: viruses which are structurally similar but differ with respect to their transcription and translation stra-

Table 1 – Present classification of viruses formerly accommodated in the Togaviridae family

| Family | Genus | Prototype | Number of viruses |
|--------------------------|-------------|--|-------------------|
| Flaviviridae | Flavivirus | Yellow fever virus | 64 |
| Togaviridae | Alphavirus | Sindbis virus | 26 |
| | Rubivirus | Rubella virus | 1 |
| | Pestivirus | Mucosal disease/bovine virus diarrhoea | 2 |
| | Arterivirus | Equine arteritis virus | 1 |
| not assigned to genera : | | Lactic dehydrogenase virus | |
| | | Simian haemorrhagic fever virus | |

tegies cannot belong to the same taxonomic cluster. Let us review the togaviruses in this respect: alphaviruses produce two mRNAs after infection; one is identical to the virion RNA, and its 5'-part is translated into the non-structural (polymerase) proteins involved in virus replication. The second molecule is a subgenomic mRNA identical to the 3'-terminal third of the genomic RNA; it is translated into the structural proteins of the virion (Strauss and Strauss 1983).

The replication strategy of rubella virus is very similar to that of the alphaviruses (Oker-Blom *et al* 1983, Kaikkinen *et al* 1984). A genomic 40 S RNA and an additional 24 S RNA, identical to the 3'-end of the genomic RNA were isolated from infected cells. The subgenomic RNA encodes a precursor (p110) to the structural proteins (Oker-Blom *et al* 1984).

Establishment of the new genus arterivirus within the togaviridae (table 1) has been based on work published from the Utrecht group on the viral genome (Van der Zeijst *et al* 1975) and proteins (Zeegers *et al* 1976). We are afraid, however, that this will be an ephemeral hierarchical status since our recent results indicate a strategy of replication for EAV which is fundamentally different from that of the other family members. Analysis of the RNA synthesized in infected cells revealed the existence of six virus-specific species. The sum of the molecular weights of the subgenomic RNAs amounts to 3.4×10^6 daltons which is about 20 % less than the molecular weight of the genome. When Northern blot analyses were carried out using radiolabelled DNA probes complementary to the smallest mRNA it was shown to hybridize with all other RNAs indicating that they must possess common sequences. A similar conclusion was reached when comparing ribonuclease T1 oligonucleotide fingerprints of the different subgenomic molecules. These results support the hypothesis that the intracellular RNAs of EAV form a nested set, not unlike that in coronaviruses. Preliminary results from cloning and sequencing also suggest that they possess a common 3' terminal end.

Let us dwell a little longer on the replication of EAV. Of central importance to the study of viral gene expression are the mechanisms by which the mRNAs are produced. Upon infection a negative-stranded RNA is synthesized from the parental RNA, which serves as the template for novel positive-stranded RNA synthesis. Until now two mechanisms by which subgenomic RNAs can arise are known for positive-stranded RNA viruses:

- i) internal initiation by the RNA-polymerase on the negative strand of genomic RNA (eg alphaviruses, Kennedy 1980, rubella virus, Oker-Blom 1984)
- ii) fusion of leader and body sequences, which are non-contiguous in the genome and are joined in the cytoplasm (eg coronaviruses, Spaan *et al* 1983, Lai *et al* 1984). A number of plant viruses have structural features in common with plus sense animal viruses and also share similarities in their replication strategies (Haseloff *et al* 1984, Ahlquist *et al* 1985). They also use different ways to produce subgenomic messenger RNAs (Joshi and Haenni 1984), one of them involving premature termination during negative strand synthesis, followed by independent replication of the subgenomic negative strand (Goelet and Karn 1982). Nucleolytic cleavage has been suggested as another possibility for subgenomic RNA production (Gonda and Symons 1983), and probably occurs during EAV replication.

Sequence rearrangements can be expected if several cleavage products are fused. Although we have no direct evidence for splicing in EAV, the anomalies detected in the T1 fingerprints of individual mRNAs suggest that rearrangements do exist. However, splicing has only been found to happen in the nucleus, and in EAV replication nuclear functions have not unequivocally been identified. Although a universal mechanism does not seem to be involved, highly conserved sequences located at the intron/exon junctions are required for accurate and efficient splicing (Rogers 1985). Data on the nucleotide sequence of the

genome and of the subgenomic RNAs are presently collected to unravel the details of the transcription of EAV.

From these data we feel that EAV can no longer be accommodated in the togavirus family. Irrespective of similarities in virion structure, its different replication mechanism brings EAV to the same hierarchic level as that occupied by the coronaviridae which is the family level. We have discussed replication in some detail to indicate how important it may be for virus classification. Recently, the flaviruses have been elevated to the family status. In flavivirus infected cells subgenomic RNA has not been found and the general opinion is that the viral genome is the only messenger. The entire genome of yellow fever virus has now been sequenced (Rice *et al* 1985); the sequence reveals a single open reading frame (ORF) of 10 233 nucleotides, which could encode a polypeptide of 3 411 amino acids. The structural proteins are found within the amino-terminal 780 residues of this polypeptide. The 5'-location of the genes encoding the structural proteins, the single long ORF, and the lack of a subgenomic message are characteristics shared with picornaviruses rather than with togaviruses (for a review see Strauss and Strauss 1983).

The replication strategy of the pestiviruses has been even less studied. Only on BVDV some results have been published by Purchio *et al* (1983, 1984a, 1984b). In infected cells a single species of virus specific RNA with a mol wt of 2.9×10^6 has been found. *In vitro* translation of this RNA resulted in the synthesis of several polypeptides, which could be immunoprecipitated using a virus specific antiserum. None of the polypeptides synthesized *in vitro* appeared to co-migrate with authentic viral proteins immunoprecipitated from infected cells. Recently, however, the genome of BVDV has been cloned; its sequence revealed two non-overlapping ORFs (Renard *et al* 1986). We will hear more about this during the present meeting. The workers from Liege have determined the length of the BVDV genome to be 12.5 kb corresponding to 4.4×10^6 daltons. This value is at variance with the data of Purchio *et al* (1983) but confirm our earlier estimates, using the infectivity of extracted RNA, a good criterium for its

undegraded state (Moennig 1971 cited from Horzinek 1981). Although the pestiviral genome appears to be about 25 % larger than that of the flaviruses the similarities are striking: pestiviral genomic RNA is also not polyadenylated and the structural virion proteins are encoded by sequences at the 5'-end with the capsid protein first, followed by (glycosylated) envelope proteins (Renard *et al* 1986). When trying to answer the question which constitutes the title of this paper one might say that pestiviruses belong in the neighbourhood of flaviviruses, which actually has been suggested already in 1963 (Dinter 1963). They are clearly quite different from alphaviruses and since a separate family status has been established for flaviviruses then the pestiviruses might be assigned a generic status in that family rather than in the togaviridae.

It is a logical consequence of the methodological developments during the recent years that virologists experienced in molecular biology have entered the field of pestiviruses. As can be seen from the program of the present meeting DNA recombinant technology and monoclonal antibodies have been used to explore the terra incognita. It is expected that the forthcoming years will bring the answer to many questions that have puzzled virologists for decennia eg the immunizing properties of the «soluble» antigen found in infected tissue (for discussion see Horzinek 1981), its relationship to chymotrypsin (Matthaeus and Korn 1975), the unequivocal identification of the nucleocapsid and envelope proteins, their (lack of) relationship with the «soluble» antigens, antigenic and host range variation of pestiviruses, etc. A major single breakthrough would be the preparation of a safe and potent vaccine against pestivirus infections in pigs and cattle; in view of the notorious difficulties to achieve this goal by conventional means, this is a challenge for the cloners and sequencers amongst us. If this operation were successful it would not only boost the waning morale of venture capital investors in biotechnology; also the worldwide community of frustrated pestivirologists would at last experience the solution of puzzles which many of them have despaired to solve.

Abstract

In this introduction to the meeting, the author specifies the place of pestiviruses among RNA viruses and briefly summarizes the main characteristics from this genus.

References

AHLQUIST P, STRAUSS EG, RICE CM, STRAUSS JH, HASELOFF J, ZIMMERN D, 1985. Sindbis virus proteins nsP1 and nsP2 contain homology to nonstructural proteins from several RNA plant viruses. *J Virol* 53:536-542

- ANDREWES CH, 1970. Generic names of viruses of vertebrates. *Virology* 40:1070-1071
- DINTER Z, 1963. Relationship between bovine virus diarrhea and hog cholera virus. *Zentralbl Bakteriell Parasitenkd I* 188:475-486
- GOELET P, KARN J, 1982. Tobacco mosaic virus induces the synthesis of a family of 3' coterminal messenger RNAs and their complements. *J Mol Biol* 154:541-550
- GONDA TJ, SYMONS RH, 1983. Cucumber mosaic virus replication in cowpea protoplasts: time course of virus, coat protein and RNA synthesis. *J Gen Virol* 45:723-730
- HASELOFF J, GOELET P, ZIMMERN D, AHLQUIST P, DASGUPTA R, KAESBERG P, 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. *Proc Natl Acad Sci USA* 81:4358-4362
- HORZINEK MC, 1973a. Comparative aspects of togaviruses. *J Gen Virol* 20:87-103
- HORZINEK MC, 1973b. The structure of togaviruses. *Progr Med Virol* 16:109-156
- HORZINEK MC, 1981. Non-arthropod-borne togaviruses. Academic Press, London
- HORZINEK MC, WEISS M, 1984. Toroviridae: a taxonomic proposal. *Zentralbl Veterinaermed B* 31:649-659
- HORZINEK MC, WEISS M, EDERVEEN J, 1984. Berne virus is not "coronavirus-like". *J Gen Virol* 65:645-649
- HORZINEK MC, EDERVEEN J, WEISS M, 1985. The nucleocapsid of Berne virus. *J Gen Virol* 66:1287-1296
- HORZINEK MC, EDERVEEN J, KAEFFER B, DE BOER D, WEISS M, 1986. The peplomers of Berne virus. *J Gen Virol*, 67:2475-2483
- JACOBS L, SPAAN WJM, HORZINEK MC, VAN DER ZEIJST BAM, 1981. Synthesis of subgenomic mRNAs of mouse hepatitis virus is initiated independently: evidence from UV transcription mapping. *J Virol* 39:401-406
- JOSHI S, HAENNI A, 1984. Plant RNA viruses: strategies of expression and regulation of viral genes. *FEBS Letters* 177:163-174
- KALKKINEN N, OKER-BLOM C, PETERSON RF, 1984. Three genes code for rubella virus structural proteins E1, E2a, E2b and C. *J Gen Virol* 65:1549-1557
- KENNEDY SIT, 1980. Synthesis of alphavirus RNA. In Schlesinger R W (ed). *The Togaviruses* p. 351-369, Academic Press London
- KOOPMANS M, EDERVEEN J, WOODS GN, HORZINEK MC, 1986. The surface proteins of Breda virus. *Am J Vet Res* 47:1896-1900
- LAI MM, BARIC RS, BRAYTON PR, STOHLMAN SA, 1984. Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic RNA virus. *Proc Natl Acad Sci USA* 81:3626-3630
- MATTHAEUS W, KORN G, 1975. Das Verhalten von präzipitierenden Substanzen aus normalen Organen und Zellen im Vergleich zu dem der präzipitierenden Pankreas-antigene mit Schweinepest infizierter Schwein. *Zentralbl Veterinaermed B* 22:239-253
- OKER-BLOM C, KALKKINEN N, KAARIAINEN L, PETERSON RF, 1983. Rubella virus contains one capsid protein and three envelope glycoproteins, E1, E2 and E2b. *J Virol* 46:964-973
- OKER-BLOM C, ULMANEN I, KAARIAINEN L, PETERSON RF, 1984. Rubella virus 40S genome RNA specifies a subgenomic 24S mRNA that codes for a precursor to structural proteins. *J Virol* 49:403-408
- PURCHIO AF, LARSON R, COLLETT MS, 1983. Characterization of virus-specific RNA synthesized in bovine cells infected with bovine viral diarrhea virus. *J Virol* 48:320-324
- PURCHIO AF, LARSON R, COLLETT MS, 1984a. Characterization of bovine viral diarrhea virus proteins. *J Virol* 50:666-669
- PURCHIO AF, LARSON R, TORBORG LL, COLLETT MS, 1984b. Cell-free translation of bovine viral diarrhea virus RNA. *J Virol* 52:973-975
- RENARD A, BROWN-SHIMMER S, SCHMETZ D, GUIOT C, DAGENAIS L, PASTORET PP, DINA D, MARTIAL J, 1986. Molecular cloning, sequencing and expression of BVDV RNA. In press
- RICE CM, LENCHES EM, EDDY SR, SHIN SJ, SHEETS RL, STRAUSS JH, 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* 229:726-733
- ROGERS JH, 1985. The origin and evolution of retroposons. Part 1: Mechanisms of RNA splicing. *Intern Rev Cytol* 93:187-231
- SPAAN WJM, ROTTIER PJM, HORZINEK MC, VAN DER ZEIJST BAM, 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). *Virology* 108:424-434
- SPAAN W, DELIUS H, SKINNER M, ARMSTRONG J, ROTTIER P, SMEEKENS S, VAN DER ZEIJST BAM, SIDDELL SG, 1983. Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. *EMBO J* 2:1839-1844
- STERN DF, KENNEDY SIT, 1980. Coronavirus multiplication strategy. 2. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. *J Virol* 36:440-449
- STRAUSS EG, STRAUSS JH, 1983. Replication strategies of the single stranded RNA viruses of eukaryotes. *Curr Top Microbiol Immunol* 105:1-98
- VAN DER ZEIJST BAM, HORZINEK MC, MOENNIG V, 1975. The genome of equine arteritis virus. *Virology* 68:418-425

- WEISS M, HORZINEK MC, 1986. Morphogenesis of Berne virus (proposed family Toroviridae). *J Gene Virol* 67:1305-1314
- WEISS M, STECK F, HORZINEK MC, 1983. Purification and partial characterization of a new enveloped RNA virus (Berne virus). *J Gen Virol* 64:1849-1858
- WOODE GN, REED DE, RUNNELS PL, HERRIG MA, HILL HT, 1982. Studies with an unclassified virus isolated from diarrheic calves. *Vet Microbiol* 7:221-240
- ZEEGERS JJW, VAN DER ZEIJST BAM, HORZINEK MC, 1976. The structural proteins of equine arteritis virus. *Virology* 73:200-205