Multiple Homologies of Oligonucleotide Size Exist between Nucleic Acids of Picornaviruses

By O. MARQUARDT 1* AND P. A. BACHMANN 2

1 Max-Planck-Institut für Biochemie, 8033 Martinsried, and 2 Institut für Medizinische Mikrobiologie, Infektions- und Seuchenmedizin, 8000 München 22, Federal Republic of Germany

(Accepted 7 February 1983)

SUMMARY

A semi-quantitative analysis of hybrid formation between restriction enzyme-generated subgenomic fragments of cloned cDNA prepared from RNA of foot-and-mouth disease virus (FMDV) strain O1K and radiolabelled RNA from bovine enterovirus, bovine rhinovirus or Mengo virus indicated that the hybrids were of oligonucleotide size. They were located in those parts of the FMDV O1K genome that code for the two capsid proteins VP3 and VP1 and the precursor protein P52 as well as at the 3'end. No hybridization was observed with poliovirus type 1 RNA.

Picornaviruses (for review, see Cooper et al., 1978) consist of a single-stranded RNA molecule 7000 to 8000 bases long of (+) polarity which is encapsidated by 60 copies of each of the four capsid proteins. The primary product of protein synthesis is a precursor polyprotein from which the mature proteins are released after extensive processing. The aphthoviruses represent one of the four genera grouped in this virus family. They can be distinguished from the others (enteroviruses, rhinoviruses and cardioviruses) by their different buoyant densities in caesium chloride, stabilities in the pH range 3 to 7 or ribonucleotide compositions. The 3'-proximal RNA sequences of aphthoviruses are highly homologous, an observation providing evidence for the close relationship of the serotypes. The 5'-proximal sequences coding for the capsid proteins VP1 and VP3 are less homologous (Marquardt, 1982); this may explain the antigenic variation between the serotypes noticed some time ago (Galloway et al., 1948; Wild et al., 1969).

The specific distribution of sequence homology among aphthoviruses made us consider whether sequence homologies exist between distinct genome areas of aphthoviruses and those of other picornaviruses. The availability of bacterial clones which contain foot-and-mouth disease virus (FMDV) O1K-specific complementary DNA (Küpper et al., 1981) facilitated such studies. The experimental strategy is based on the correlation of the cloned cDNA to the map of FMDV-induced polypeptides (Fig. 1). It takes advantage of restriction enzymes which cleave FMDV-specific double-stranded DNA into defined subgenomic fragments. These fragments are analysed individually as to whether or not they hybridize with radiolabelled RNA by use of the technique of Southern (1975). Since RNA that did not hybridize is removed by RNase A digestion, the remaining RNA/DNA hybrids consist of true double-strands. They require a minimum size between more than 6 and about 30 base pairs (bp) to be detectable.

Such a technique which detects homologies of oligonucleotide size is required to reveal common picornavirus sequences, because a study comparing the RNase T1 digestion products of the RNAs of representative members of three genera (Frisby et al., 1976) did not find large common oligonucleotides. However, a pentanucleotide resistant to the RNases T1 and A was shown to be shared by at least four RNAs. This could form the core of a common oligonucleotide consisting of more than six bases. The only homology thus far reported comprises 15 out of 19 nucleotides including a decanucleotide. It is observed in the sequenced 3'-termini of encephalomyocarditis virus and poliovirus type 1 RNA (Porter et al., 1978).

The comparative study presented here included members of all genera. Two were bovine non-aphthoviruses [bovine enterovirus (ECBO = BEV) and bovine rhinovirus (BRV)], and two
Fig. 1. Correlation of FMDV proteins to FMDV cDNA and the location of oligonucleotide homology with RNA of other picornaviruses. The map of FMDV-induced polypeptides (a) (Sangar, 1979) was correlated to the restriction map of cloned cDNA from FMDV O1K (b) (Küpper et al., 1981) using the precise location of the coding sequence of VP1 (Kurz et al., 1981). The length of the cDNA is given in kilobases (kb) beginning with the 3' end. Cloned cDNA contains oligo(dG-dC) tracts of about 30 bp (g). The relevant cleavage sites for restriction enzymes (B, BamHI; E, EcoRI; H, HindIII) and the resulting FMDV-specific DNA fragments (2, 4, 6, 7, 8, 9 and 12) are indicated. The fragments which hybridize to bovine enterovirus, bovine rhinovirus and Mengo virus RNA, as shown in Fig. 2(c to e), are designated BEV, BRV and MV respectively.

others were non-bovine picornaviruses (Mengo virus and poliovirus type 1, Mahoney). We present data obtained by a simple and quick approach which show for the first time that hybrids of oligonucleotide size are formed between FMDV O1K-specific DNA and RNA from BEV, BRV or Mengo virus. However, no homology is observed with RNA from poliovirus. We show further that the observed homologies are scattered all over the compared parts of the genomes except that which codes for the FMDV precursor protein P56c.

The FMDV O1K-specific DNA of the four plasmids pFMDV1448, pFMDV849, pFMDV703 and pFMDV512 used in this study represents contiguous stretches covering 70% of the virus genome including the 3' end (Fig. 1). The 1600 bp insert of pFMDV1448 mainly contains codons for the two capsid proteins VP3 and VP1. The 2200 bp insert of pFMDV849 contains the codons for the precursor protein P52 and the N-terminal part of protein P56c. The 2400 bp insert of pFMDV703 comprises the codons for the proteins P20 and P56c and half of the codons for protein P56a. The 1200 bp insert of pFMDV512 represents the 3'-terminal part of the RNA. The plasmids were harvested under standard conditions and submitted to restriction endonuclease digestions as recommended by the suppliers (BRL, Renner, F.R.G.). An agarose gel analysis of these reactions is shown in Fig. 2(a). The inserts of pFMDV1448 (lane 1) and pFMDV512 (lane 4), flanked by oligo(dG-dC) tracts of approx. 30 bp each (Kurz et al., 1981), originating from the technique used for DNA recombination, were released from the vector DNA (pBR322) by digestion with the enzyme PstI; pFMDV849 (lane 2) was digested with PstI and HindIII in order to generate two fragments of vector DNA as well as two FMDV O1K DNA fragments containing one distal oligo(dG-dC) tract each; pFMDV703 (lane 3) was digested with EcoRI and BamHI to give one FMDV O1K DNA fragment, one fragment of the vector DNA and two fragments consisting of vector DNA as well as FMDV O1K DNA linked by an oligo(dG-dC) tract. The number of bp per fragment and the specification is given in the insert to
Fig. 2. Hybridization results obtained with RNA of different picornaviruses and the digested plasmids (pFMDV) 1448, 849, 703 and 512 (Fig. 1). The total number of bp as well as that of FMDV O1K specificity is shown. The vector DNA (v) of known sequence (Sutcliffe, 1978) is used for the size determination of FMDV DNA (deviation being 50 bp), which contains either two oligo(dG–dC) tracts (gg), one (g) or none. It also serves as a negative hybridization control. Lane 1, pFMDV1448 DNA digested with restriction enzyme PstI into fragments 1 and 2; lane 2, pFMDV849 DNA digested with PstI and HindIII into fragments 3, 4, 5 and 6; lane 3, pFMDV703 DNA digested with EcoRI and BamHI into fragments 7, 8, 9 and 10; lane 4, pFMDV512 DNA digested with PstI into fragments 11 and 12. (a) Photograph of the ethidium bromide-stained gel in u.v. light (the position of fragment 6 is known from an autoradiograph); (b to e) autoradiographs of nitrocellulose filters corresponding to parallels of (a) after hybridization to (b) Coxs RNA (2 × 10^6 ct/min/0.2 μg; 5 h exposure), to (c) BRV RNA (3 × 10^6 ct/min/1 μg; 7 days exposure), to (d) MV RNA (3 × 10^6 ct/min/0.4 μg; 5 days exposure) and to (e) BEV RNA (2 × 10^6 ct/min/0.2 μg; 7 days exposure). Alkaline hydrolysis was always performed for 60 min at 90 °C. The position of the DNA fragments in (c) differs from that in the other panels because of a longer electrophoresis time.
BRV (strain SD 114) was grown at 33 °C in bovine embryonic kidney cell cultures. The infectivity titre of the material was $10^{5.0}$ TCID$_{50}$/0.1 ml. BEV (strain 100C) was grown at 37 °C in bovine embryonic lung cell cultures. The infectivity titre was $10^{7.5}$ TCID$_{50}$/0.1 ml. Cell cultures were prepared from kidneys and lungs of 3- to 5-month-old bovine embryos by trypsinization according to standard methods. Cells were grown in minimal essential medium with Earle's balanced salt solution (EMEM) and 5% foetal calf serum (FCS) in roller bottles. The virus medium used was EMEM with 1% FCS. Both virus preparations were harvested at maximum cytopathic effect and frozen at $-70$ °C. After two cycles of freezing and thawing the virus suspensions were filtered. The viruses were concentrated and their RNA was prepared as described by Strohmaier & Adam (1976).

Each of the $^{32}$P-labelled RNAs served as a probe in hybridization experiments with the DNA fragments bound to nitrocellulose filters. Qualitative results were obtained in the form of autoradiographs of the RNase A-treated and cleaned filters (experimental details are given in Marquardt, 1982) and are shown in Fig. 2. The sequence homology of FMDV Cobb RNA with FMDV OIK DNA had been studied earlier and served here as a positive control (Fig. 2b). Indeed, $^{32}$P-labelled Cobb RNA is retained by all seven OIK DNA fragments on the filter, the degree of retention depending as expected on the degree of homology (compare fragments 2 and 12 in Fig. 2b). The signal from fragment 6 could only be seen after longer exposure of the autoradiograph (not shown), indicating a suboptimal transfer of this DNA to the filter, as is frequently observed with small fragments (Southern, 1975). No radiolabelled RNA is bound to vector DNA (fragments 1, 3, 5, 10 and 11) here or in the other experiments (Fig. 2b to e), which proves the specificity of the results. They show that RNAs prepared from two bovine viruses (BRV and BEV) and a non-bovine virus (Mengo) contain sequences which hybridize to an aphthovirus cDNA (Fig. 2c to e respectively). When such an experiment was done with radiolabelled poliovirus RNA ($1 \times 10^7$ or $7 \times 10^7$ ct/min/µg) no hybridization signal could be detected (data not shown).

Since the order of genes is the same in all picornavirus genomes (Cooper et al., 1978), the results show further that the homologies are located within the capsid protein genes (VP1 and VP3, fragment 2), the adjacent precursor protein gene (P52, fragment 4) and the 3' ends (fragment 12, Fig. 1). The latter result cannot be attributed solely to possible hybrid formation between the poly(A) tracts present in virus RNA and the complementary cloned poly(dT) tract, as fragment 9 alone, which is a subfragment of fragment 12 (Fig. 1), elicits autoradiographic responses (Fig. 2c, e). Further evidence that the homologies observed with fragment 12 probably lie in the coding sequences comes from the heterology detected comparing the sequenced 3'-termini of five FMDV strains with those of two enteroviruses and two cardioviruses (Porter et al., 1978). The hybridization pattern obtained with Mengo virus RNA (Fig. 2d) differs from those obtained with BRV or BEV RNA (Fig. 2c, e) in fragments 7 (positive) and 9 (negative). Fragment 7 codes for protein P20, the function of which is not known. Hybridization is never observed with fragment 8 (Fig. 2c to e). It codes for most of the precursor protein P100 from which the RNA-dependent RNA polymerase, the protease and the peptide VPg are released. The comparison of FMDV and poliovirus nucleic acid sequences coding for VPg (Forss & Schaller, 1982) reveals a homologous pentanucleotide and some di- and trinucleotides. Since they are separated by heterologies, they cannot be detected by the technique used in this study. However, comparing the corresponding amino acid sequences, a relationship of these peptides is obvious.
The implication of this finding for the data presented here is twofold. Failure in detecting nucleic acid homology by hybridization does not imply that the amino acid sequences are unrelated. This can be explained by the degeneracy of the genetic code. On the other hand, if there is no evolutionary need for picornavirus genes and those of virtually all RNA viruses (Holland et al., 1982) to be considerably conserved, then the observed conservation of certain coding sequences most likely indicates that mutations here are disadvantageous. Apart from serving as codons such sequences may be important as signals at the nucleic acid level (e.g. for packaging).

Southern blot hybridizations can be analysed semi-quantitatively because the retention of RNA by homologous complementary DNA bound to nitrocellulose is a function of the DNA fragment size. By comparing the capacity of the same DNA fragments to hybridize with heterologous RNA under stringent conditions, the degree of homology of both RNA species can be estimated (Marquardt, 1982). The homologies shown in Fig. 2 were analysed by first cutting each filter into strips corresponding to tracks in the gel and fractionating the strips. The RNA-bound radioactivity in each fraction was counted in a scintillation spectrometer and compared to that in the corresponding fraction from an experiment where $^{32}$P-labelled FMDV O1K RNA was hybridized to a filter (autoradiogram not shown). This analysis reveals that the hybrids formed between FMDV O1K DNA and BRV, Mengo virus or BEV RNA never exceed 1% of the possible homology, whereas those containing C$_{obs}$ RNA agree with the published results (data not shown). The size of the hybrids formed between heterologous nucleic acids (Fig. 2c to e) was estimated as follows. It can be seen (Fig. 2b, fragment 9) that there is enough sequence complementarity within a sequence of 60 bases to hybridize sufficient radiolabelled RNA for an autoradiographic response to be elicited. The radioactivity measured in this filter fraction was 54 ct/min over background (neighbour fractions), indicating 60 to 80% sequence homology. The value was higher than, for example, those measured for the fragments 2 in Fig. 2c to e. The hybrids must therefore be considerably smaller than 36 to 50 bp, that is the size of oligonucleotides.

The lack of detectable sequence homology between FMDV and poliovirus in hybridization experiments shows that picornaviruses do not need short common oligonucleotides. Since the technique applied underestimates the overall sequence homology, sequence similarities cannot be detected. Apart from the similar poliovirus and FMDV VPg genes (Forss & Schaller, 1982) other similarities are suggested by the oligonucleotide homologies observed between poliovirus and encephalomyocarditis virus (Porter et al., 1978) and another enterovirus (BEV) and FMDV, but will be provable only by comparing complete nucleic acid sequences. The result obtained with Mengo virus provides further evidence for a phylogenetic relationship of cardioviruses and aphthoviruses. These viruses contain a genomic poly(C) tract, in contrast to the other picornaviruses (Brown et al., 1974), and a comparison of partial capsid protein sequences reveals homologies between FMDV VP2 (its gene not being included in this study) and its Mengo virus equivalent (Strohmaier et al., 1978), which is discussed as strong evidence for relationship. Human rhinoviruses lack close similarities in nucleic acid sequence (Yin et al., 1973) and nothing is known about homologies between BRVs or human and bovine rhinoviruses. Furthermore, it is not known either whether the BRV strain used here may be considered as a representative example for the genus. The lack of knowledge about rhinovirus nucleic acids justifies the acquisition of any further information, especially one indicating sequence homology to FMDV O1K, a member of another picornavirus genus.

We hope that these data will stimulate further studies on picornaviruses such as BRV, BEV and Mengo virus, so that a number of picornavirus nucleotide sequences can be compared, allowing for a better understanding of this virus family.

We are grateful to Drs R. Dernick, K. Strohmaier, K. Wetz and V. Zaslavsky for supplying us with virus RNA and to Dr P. H. Hofschneider for the interest he took in this work. We further wish to thank Miss M. Wechsler for excellent technical assistance.
REFERENCES


(Received 14 October 1982)