INFECTION AND IMMUNITY, Apr. 1982, p. 142–147
0019-9567/82/040142-06$02.00/0

Vol. 36, No. 1

Application of RNase T_1 One- and Two-Dimensional Analyses to the Rapid Identification of Foot-and-Mouth Disease Viruses

J. L. LA TORRE,† B. O. UNDERWOOD, M. LEBENDIKER,† B. M. GORMAN,‡ AND F. BROWN*

Animal Virus Research Institute, Pirbright, Woking, Surrey, United Kingdom

Received 21 September 1981/Accepted 1 December 1981

The analysis of several isolates of foot-and-mouth disease virus by RNase T_1 fingerprinting of the ^{32}P-labeled RNA is described. It has been shown that use of the 35S induced RNA instead of the virus particle RNA has two advantages. (i) About 40 times more radioactivity is incorporated into the induced RNA. (ii) The RNA can be prepared much more rapidly, thus increasing the value of the technique in rapid diagnosis. One-dimensional maps, in which the RNase T_1 oligonucleotides are separated according to size, have been shown to provide a valuable screening method for distinguishing between viruses. Those viruses giving similar one-dimensional maps also gave similar two-dimensional maps. The value of using the length of the polycytidylic acid tract of foot-and-mouth disease virus as a diagnostic tool is also discussed.

RNase T_1 fingerprinting has been used for the identification and differentiation of isolates of many different families of viruses (3). It has found application in studying the epidemiology of the diseases caused by these viruses and has proved valuable, for example, in the rapid identification of the virus causing recent outbreaks of foot-and-mouth disease in France and the United Kingdom (10a). It became apparent during this investigation that more rapid methods for characterizing the virus would be valuable. In consequence, we decided to investigate whether the 35S induced RNA could be used instead of the RNA of the virus particle. This would obviate the need to isolate the virus particles and would have the additional advantage of providing more material because we had shown previously that not more than 20% of the 35S induced RNA is incorporated into virus particles (16).

It was also considered that further speeding of the analysis would be achieved if a rapid screening for nonidentity of the RNAs were made by one-dimensional (one-D) separation of the RNase T_1 hydrolysates according to the size of the oligonucleotides. The value of this type of analysis is described.

Foot-and-mouth disease virus (FMDV), like the cardioviruses, contains a polycytidylic acid [poly(C)] residue near the 5′ end of its genome (2, 5, 9, 12). The position of the tract is the same for all of the FMDV isolates we have examined so far, but the length varies considerably between isolates. This provides another method for distinguishing between viruses which otherwise give very similar RNase T_1 two-dimensional (two-D) maps.

Application of these methods to the analysis of several FMDV isolates of current epidemiological interest is described, and the value of a library of biochemical profiles of FMDV as an aid to their rapid identification is discussed.

MATERIALS AND METHODS

Viruses. FMDV, serotype A, subtype 10 (strain A61), was used for determining the conditions necessary for the different biochemical methods. The following viruses of serotype O were then compared by these methods: United Kingdom (UK) 1848 and 18/81, Lausanne 1965, India 53/79, and Thailand 1/80. All of the viruses were obtained from the World Reference Laboratory at the Animal Virus Research Institute and are named according to its reference system.

Preparation of ^{32}P-labeled virion RNA. Roux bottles containing approximately 10^8 BHK-21 cells were infected at a multiplicity of 10 to 100. After 45 min the virus was removed, and the sheets were washed with prewarmed phosphate-free medium (three changes during 1 h at 37°C) (11). After the last wash medium containing 3 μg of actinomycin D per ml was added. At 2 h postinfection (p.i.) 200 μCi of [^{32}P]orthophosphoric acid per ml were added (carrier-free from the Radiochemical Centre, Amersham, United Kingdom). Virus was harvested at 5 h p.i. and purified (4, 6), and the RNA was extracted from the sucrose gradient fractions as described previously (6, 9).

Preparation of ^{32}P-labeled RNA from the cytoplasm of infected cells. BHK cell monolayers were infected as described above. Cytoplasmic extracts were made

† Present address: Centro de Virologia Animal, Serrano 661, 1414 Capital Federal, Argentina.

‡ Present address: Queensland Institute for Medical Research, Brisbane, Australia.

when most of the cells were rounded and ready to leave the glass, which was usually between 3 and 6 h p.i., depending on the strain of virus used. The medium was poured off, and the cells were disrupted in TNE buffer (0.05 M Tris [pH 7.5], 0.14 M NaCl, and 0.001 M EDTA containing 0.05% Triton X-100) (15). Bottles were shaken vigorously for 5 min in the cold, transferred to 20-ml plastic bottles having a conical bottom, and centrifuged for 3 min at 1,000 × *g* to pellet the nuclei. Supernatants were made 1% with respect to sodium dodecyl sulfate, and the RNA was extracted twice with phenol-chloroform. The RNA was precipitated from the aqueous phase by the addition of 2 volumes of ethanol, kept for at least 2 h at −70°C, dissolved in TNE buffer, and reprecipitated with an equal volume of 4 M LiCl for at least 12 h at 4°C (1).

Sucrose gradient centrifugation of RNA. Ethanol precipitates of virion or induced RNA were separated by centrifugation, washed three times with cold ethanol, and redissolved in TNE buffer, pH 7.5, containing 0.2% sodium dodecyl sulfate. Each sample was layered onto a gradient of 10 to 30% (wt/vol) sucrose in TNE buffer and centrifuged at 60,000 × *g* for 12 h at 23°C in the Beckman SW28 rotor. Fractions (1 ml each) were collected, and the radioactivity in each fraction was assayed by the Cerenkov effect of a 10-μl sample, or alternatively, by drying 10-μl samples on glass fibre disks and counting in a scintillation fluid of toluene–2,5-diphenyloxazole (PPO)–1,4-bis-(5-phenyloxazolyl) benzene (POPOP) with a Packard spectrometer. Peaks of radioactivity sedimenting at approximately 35S were pooled, diluted with an equal volume of TNE buffer, and precipitated with 2 volumes of ethanol at −20°C for 18 h.

Cellulose chromatography of induced RNA. A 10-μl sample containing approximately 60,000 cpm was diluted in 200 μl of TNE–15% ethanol and passed through a CF11 column (7). The filtrate was precipitated with 2 volumes of ethanol at −20°C for subsequent analysis.

Enzymatic digestion of RNA with RNase T_1. RNA precipitates were centrifuged, dissolved in 0.3 M acetate buffer, pH 5.0, transferred to Eppendorf tubes, and reprecipitated with ethanol at −70°C for 1 h. These precipitates were collected by centrifugation at 17,000 × *g* for 14 min, dried in vacuo, redissolved in T1 buffer (0.02 M Tris–0.002 M EDTA, pH 7.6), and digested for 1 h at 37°C with RNase T_1 (Sankyo) at an enzyme/substrate ratio of 1:20. An equal volume of dye marker mixture was added (6 M urea, 3 M sucrose in water containing 15 mg of *Escherichia coli* tRNA per ml, and 0.2% each of bromophenol blue and xylene cyanol).

Separation of oligonucleotides by two-D electrophoresis. The method was that described by Frisby et al. (8) with minor modifications.

Separation of oligonucleotides by one-D electrophoresis. RNase T_1 digests were precipitated with ethanol at −70°C for 60 min. Precipitates were deposited by centrifugation in an Eppendorf centrifuge for 14 min at 17,000 × *g*, dried in vacuo, and dissolved in 2 μl of dye marker solution containing 80% (vol/vol) deionized formamide. Samples were then analyzed essentially as described by Sanger and Coulson (14). Gels were preelectrophoresed for 30 min before the addition of samples, which were then electrophoresed at 1,200 V and 20 mA until the bromophenol blue had run 22.5 cm and the xylene cyanol had run 12.3 cm. After electrophoresis the front plate was removed, and the gel was covered with Saran Wrap and placed in a Dupont cassette with Fuji X-ray film and a Cronex Lightning Plus screen. Exposure was carried out at −70°C. Samples containing 30 to 60,000 cpm gave clearly visible bands after an overnight exposure.

RESULTS

Preparation of virus and induced RNA. It was first necessary to determine whether the RNase T_1 fingerprint of virus-induced RNA was the same as that of the RNA extracted from virus particles. ^{32}P-labeled RNA was obtained from A61 virus particles as described previously (6, 9). Induced RNA was obtained from the cytoplasm of virus-infected BHK cells at different times after infection with the virus at a multiplicity of 10 to 100. The preparations of induced RNA were analyzed by sucrose gradient centrifugation. The profiles in Fig. 1b to d show that the optimal time for harvesting the culture was 3 to 4 h p.i. At this time, about 40 times as much radioactivity was found in the 35S RNA as was found in the RNA of virus particles prepared from a parallel culture (Fig. 1a).

Clearly the best time for extracting the RNA from infected cells will vary with the virus strain and the extent of its adaptation to growth in tissue culture cells. It was found with the A61 strain of FMDV that it was best to harvest the cells at a time when most of them were rounded but had not started to leave the glass. This time has been used for all other isolates of FMDV examined in this work. Assessment of the best time to harvest the cells is subjective, but it would be impractical to determine it precisely for each virus as was done for strain A61. However, by assessing visually the amount of cytopathic effect and using this as a measure of RNA synthesis, we were able to obtain sufficient radioactive RNA for our purpose with all of the isolates described in the present study.

Characterization of the induced RNA. The sucrose gradient profile of the A61-induced RNA at 4 h p.i. is shown in Fig. 1d. Chromatography on CF11 cellulose of the RNA sedimenting at ca. 35S (fractions 5 to 12) showed that it was 97% single stranded. This RNA gave an RNase T_1 fingerprint identical with that obtained previously for virus RNA (Fig. 2a and b), and a run of a mixture of induced RNA and virus particle RNA prepared for the present study did not give any extra spots (Fig. 2c). The reproducibility of the method over several years is demonstrated by the fact that the virus particle RNA fingerprint shown in Fig. 2a is taken from a previous publication from our laboratory (13). Fingerprints being obtained at present for the

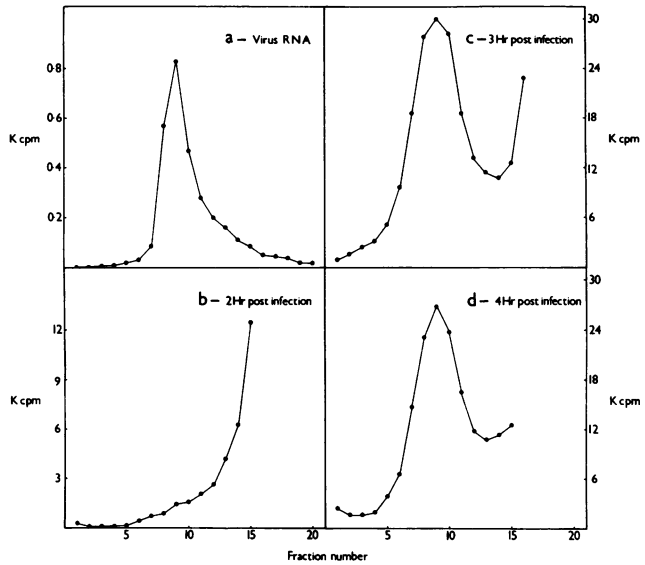

FIG. 1. Sucrose gradient centrifugation of the virus-induced RNA from FMDV-infected cells at (b) 2, (c) 3, and (d) 4 h after infection. Panel (a) shows the profile of the virus particle RNA obtained from a parallel culture.

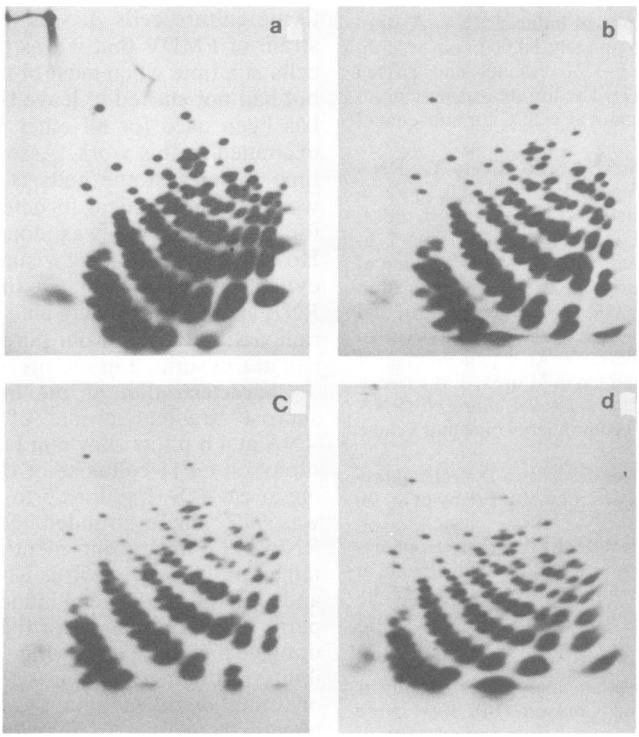

FIG. 2. RNase T_1 two-D fingerprints of (a) virus particle RNA from reference 13, (b) 35S virus-induced RNA, (c) a mixture of virus particle and virus-induced RNA, and (d) LiCl-precipitated virus-induced RNA.

A61 virus particle RNA are identical with that shown in Fig. 2a.

It was appreciated that the separation of the 35S induced RNA from the replicative intermediate and replicative form by sucrose gradient centrifugation would be time-consuming and consequently undesirable if a rapid analysis were required. Consequently, the possibility of fractionating the RNA by a quicker method was studied. It was found that the best method for our purpose was the precipitation of the total induced RNA with 2 M LiCl (1). This method was rapid, and the RNA gave a fingerprint identical with that of the 35S peak obtained from a sucrose gradient (Fig. 2d). Similar experiments with several different FMDV isolates have shown that the induced RNAs give patterns identical with the corresponding virus particle RNAs.

One-D electrophoresis of RNase T_1 hydrolysates. The original intention was to study the one-D patterns obtained when the RNA was digested with different enzymes. Preliminary experiments showed that RNase A, which cleaves the RNA at cytosine and uracil residues, gave a series of fast-running bands which were not useful for distinguishing between different isolates. RNase U2, which hydrolyzes at adenine residues, gave bands which will be useful for diagnostic purposes. In this study, however, we have concentrated our attention on the use of RNase T_1 because of the many comparisons which we are able to make with the data we have accumulated with two-D mapping of T_1 hydrolysates.

The virus strains used in this study had already been analyzed by two-D electrophoresis in our investigations to identify the viruses causing the recent outbreaks of foot-and-mouth disease in France and the United Kingdom (10a). Isolates Lausanne 1965 and UK 18/81, which were indistinguishable by two-D analysis, also gave one-D maps which could not be distinguished (Fig. 3a). However, viruses which could be distinguished by two-D fingerprints also gave different one-D patterns. Thus, isolate UK 1848, which was known from our previous work (8) to give a two-D fingerprint similar to, but distinguishable from, Lausanne 1965 and UK 18/81 (Fig. 4), gave a one-D profile with an extra band marked A in Fig. 3a. In addition, there was a band in the Lausanne 1965 and UK 18/81 isolates marked B in Fig. 3a which was not present in the UK 1848 isolate. This means that one-D analysis will distinguish even closely related RNAs.

The India and Thailand isolates, which had also been examined by two-D fingerprinting at the time of the outbreaks in France and the United Kingdom, gave patterns by both one- and two-D analysis which were readily distinguishable from each other and from the viruses already mentioned (Fig. 3b).

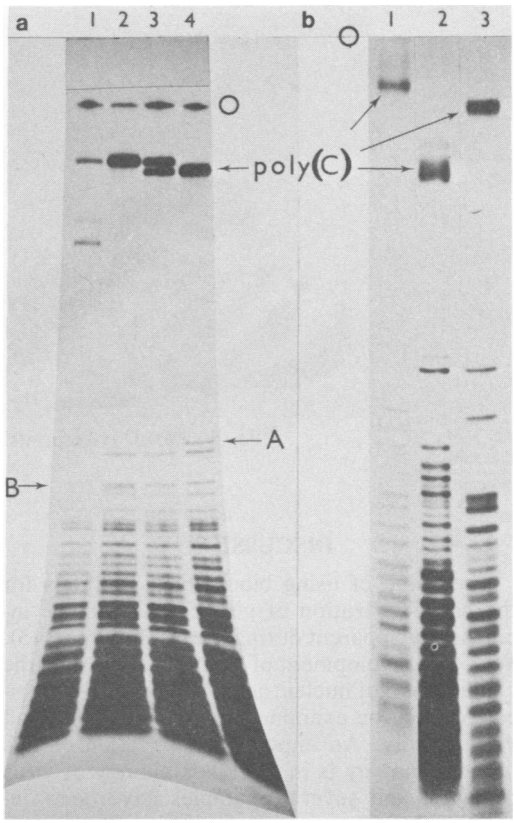

FIG. 3. RNase T_1 one-D maps of FMDV RNAs. Panel (a): (1) Lausanne 1965, (2) UK 18/81, (3) mixture of UK 18/81 and UK 1848, (4) UK 1848; panel (b): (1) India 53/79, (2) Thailand 1/80, (3) A61. O = origin. In panel (a), note the extra band marked (A) that appears for UK 1848, but not for Lausanne 1965 and UK 18/81 which, instead, exhibit the band marked B. The appearance of these bands distinguishes these closely related RNAs.

Use of the poly(C) tract in the identification of FMDV isolates. The intense band nearest to the origin of the one-D maps (shown arrowed in Fig. 3) consists of a poly(C) tract. The length of this tract varies between different virus isolates (5). The reason for this is not known, but it was found that its length was affected by repeated passage of the virus in cells (10). The value of this property was found in comparisons of the UK 1848, UK 18/81, and Lausanne 1965 isolates. The last two viruses gave identical one-D maps, and the length of the poly(C) tract in the two viruses was the same. However, the length of the poly(C) tract in the closely related UK 1848 isolate was clearly shorter.

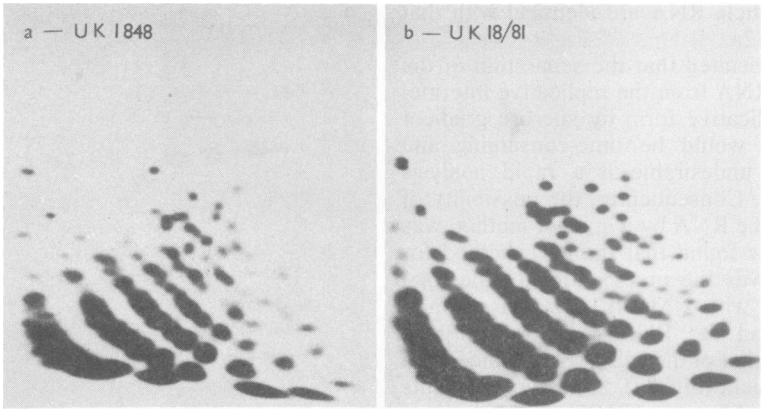

FIG. 4. Two-D fingerprints of (a) UK 1848 and (b) UK 18/81.

DISCUSSION

The value of using biochemical methods for the characterization of viruses has become increasingly apparent during the last few years (3). With the development of simple methods for the examination of nucleic acids and proteins, viruses can now be examined in considerable detail very rapidly. An important application of this new technology is in the epidemiology of virus diseases, and several examples have been described (3).

We have been particularly concerned with the epidemiology of foot-and-mouth disease and have shown the value of RNase T_1 two-D fingerprinting in the identification and differentiation of virus isolates (10a). In this paper we describe the use of one-D maps for the rapid screening of isolates so that the more complex and time-consuming two-D fingerprinting can be avoided. In addition, we have found that the 35S induced RNA and the virus particle RNA give identical fingerprints. This means that isolation of the labeled virus particles for the preparation of the virus RNA can be dispensed with and the analysis speeded up considerably.

An additional property of the RNA of FMDV which can be used for identification is the length of the poly(C) tract. Thus, the one- and two-D maps of the Lausanne 1965 and UK 18/81 isolates are indistinguishable. However, the one-D map of UK 1848, which is similar to, but distinguishable from, the maps of these two viruses, clearly has a shorter poly(C) tract. The reason for the variation in the length of the poly(C) tract of different isolates of FMDV is not known, but it is not unique to this virus because it is also found in encephalomyocarditis virus, a member of the cardiovirus genus (2). It was found that the length of the poly(C) tract in an FMDV isolate which had been passaged 82 times in BHK-21 cells was only about half of the length of that of the same virus isolate passaged 7 times in the same cells (10). Clearly, the factors underlying the change in length of the poly(C) tract require further investigation, and passage history, from the example cited above, may be of importance.

The potential value of the biochemical analysis of viruses in epidemiological studies is apparent from the present work with FMDV. A wider survey of recent foot-and-mouth disease outbreaks will provide more decisive information regarding the source of the causal viruses. The application of one-D analysis to the examination of the virus-induced RNA provides a rapid screening method which will allow a more extensive survey to be conducted.

ACKNOWLEDGMENTS

This work was done during a visit to the Animal Virus Research Institute by J.L.L., arranged under a joint agreement between the Consejo Nacional de Investigaciones Cientificas y Tecnicas of Argentina and the Royal Society of London. M.L. and B.M.G. participated in the study during short private visits to the laboratory.

LITERATURE CITED

1. **Baltimore, D.** 1966. Purification and properties of poliovirus double-stranded ribonucleic acid. J. Mol. Biol. **18**:421–428.
2. **Black, D. N., P. Stephenson, D. J. Rowlands, and F. Brown.** 1979. Sequence and location of the poly C tract in aphtho- and cardio-virus RNA. Nucleic Acids Res. **6**:2381–2390.
3. **Brown, F.** 1981. A molecular approach to the diagnosis of virus infections. Ann. Neurol. **9**(Suppl.):39–43.
4. **Brown, F., and B. Cartwright.** 1963. Purification of radioactive foot-and-mouth disease virus. Nature (London) **199**:1168–1170.
5. **Brown, F., J. Newman, J. Stott, A. Porter, D. Frisby, C. Newton, and P. Fellner.** 1974. Poly(C) in animal viral RNAs. Nature (London) **251**:342–344.
6. **Denoya, C. D., E. A. Scodeller, B. H. Gimenez, C. Vasquez, and J. L. La Torre.** 1978. Foot-and-mouth

disease virus. I. Stability of its ribonucleic acid. Virology **84**:230–235.

7. **Franklin, R. M.** 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. **55**:1504–1510.
8. **Frisby, D., C. Newton, N. H. Carey, P. Fellner, J. F. E. Newman, T. J. R. Harris, and F. Brown.** 1976. Oligonucleotide mapping of picornavirus RNAs by two-dimensional electrophoresis. Virology **71**:379–388.
9. **Harris, T. J. R., and F. Brown.** 1976. The location of the poly(C) tract in the RNA of foot-and-mouth disease virus. J. Gen. Virol. **33**:493–501.
10. **Harris, T. J. R., and F. Brown.** 1977. Biochemical analysis of a virulent and an avirulent strain of foot-and-mouth disease virus. J. Gen. Virol. **34**:87–105.

10a. **King, A. M. Q., B. O. Underwood, D. McCahon, J. W. I. Newman, and F. Brown.** 1981. Biochemical identification of viruses causing the 1981 outbreaks of foot-and-mouth disease in the United Kingdom. Nature (London) **293**:479–480.

11. **La Torre, J. L., M. J. Grubman, B. Baxt, and H. L. Bachrach.** 1980. The structural polypeptides of aphthovirus are phosphorylated. Proc. Natl. Acad. Sci. U.S.A. **77**:7444–7447.
12. **Porter, A., N. Carey, and P. Fellner.** 1974. Presence of a large poly(C) tract within the RNA of encephalomyocarditis virus. Nature (London) **248**:675–678.
13. **Robson, K. J. H., T. R. Doel, B. M. Gorman, and F. Brown.** 1980. Biochemical aspects of variation in foot-and-mouth disease virus. J. Gen. Virol. **46**:179–193.
14. **Sanger, F., and A. R. Coulson.** 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Lett. **87**:107–110.
15. **Scodeller, E., C. D. Denoya, C. Vasquez, and J. L. La Torre.** 1979. A new method for the isolation of undegraded FMDV-specific RNA from infected BHK cells. Arch. Virol. **62**:253–262.
16. **Wild, T. F., S. J. Martin, and F. Brown.** 1968. A study of the heterogeneous 37S ribonucleic acid induced by foot-and-mouth disease virus. Biochem. J. **107**:395–401.