Synthesis of Bunyavirus-specific Proteins in a Continuous Cell Line (XTC-2) Derived from Xenopus laevis

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SUMMARY

The XTC-2 cell line, derived from Xenopus laevis, supported the replication of representative viruses from each of the four genera in the family Bunyaviridae. Generally, viral titres were higher in XTC-2 cells than in other susceptible cell lines, and for some viruses plaques were detected earlier in XTC-2 cells. The XTC-2 cell line permitted comparative analyses of bunyavirus-specific protein synthesis. The patterns of synthesis of viral proteins, characteristic of each of the genera, were observed with representative viruses. These studies provided biochemical characterization of two Scottish isolates, which support the inclusion of Clo Mor virus in the Nairovirus genus and St Abb's Head (M349) virus in the Uukuvirus genus.

INTRODUCTION

More than 200 arthropod-borne viruses are classified into the family Bunyaviridae. The viruses are characterized by a tripartite, single-stranded RNA genome of negative polarity. On the basis of serological relationships and more limited biochemical analyses, four genera of Bunyaviridae have been established: Bunyavirus, Nairovirus, Phlebovirus and Uukuvirus (for reviews, see Bishop & Shope, 1979; Bishop et al., 1980). With a view to studying the genetic diversity within the family we undertook an analysis of the patterns of protein synthesis in bunyavirus-infected cells. In order to standardize such comparisons, we sought a cell line that was permissive for representative viruses from all four genera. A number of mammalian and avian cells, including BHK, BSC-1, Vero and primary chick embryo fibroblasts were tested, but none of these cell types was permissive for viruses from all genera. Previously, Leake et al. (1977) had shown that an amphibian cell line, XTC-2, derived from Xenopus laevis (Pudney et al., 1973) supported the plaque titration of a wide range of arboviruses including bunyaviruses, alphaviruses, flaviviruses and rhabdoviruses. Among the viruses used by Leake et al. (1977) were representatives of three genera within the Bunyaviridae, including Bunyamwera virus (Bunyavirus genus), Dugbe virus (Nairovirus genus) and Sicilian sandfly fever virus (Phlebovirus genus). Subsequently, Nuttall et al. (1981) used XTC-2 cells to isolate an Uukuniemi-like virus (Uukuvirus genus) from a suckling mouse brain which had been inoculated with an homogenate of infected ticks (Ixodes uriae). Since Bunyaviridae from all four genera had thus been shown to induce plaque formation in XTC-2 cells, and because XTC-2 cells were suitable for examination of intracellular viral protein synthesis (Elliott et al., 1979), we introduced this cell line into our study. This afforded the opportunity to characterize the proteins of two Scottish Bunyaviridae isolates and we present biochemical data which support the inclusion of Clo Mor virus (Main et al., 1976) in the Nairovirus genus and St Abb's Head (M349) virus (Nuttall et al., 1981) in the Uukuvirus genus.

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METHODS

Cells. XTC-2 cells were maintained at 31 °C in L-15 medium (Leibovitz, 1963) supplemented with 10% tryptose phosphate broth and 10% foetal calf serum, as described previously (Leake et al., 1977; Elliott et al., 1979). BHK, Vero and BSC-1 cells were maintained in Glasgow minimal essential medium (GMEM) containing 10% calf serum. Chick embryo fibroblasts (CEF) were prepared from 12-day-old embryos and propagated in GMEM containing 10% calf serum.

Viruses. Batai virus, Bunyamwera virus, Cache Valley virus, Guaroa virus, La Crosse virus, Maguari virus and Northway virus were obtained from Dr N. Karabatsos, Vector-borne Diseases Laboratory, Center for Disease Control, Fort Collins, Co., U.S.A.; Clo Mor virus, Kairi virus, Main Drain virus and Sathuperi virus from Dr R. E. Shope, Yale Arbovirus Research Unit, New Haven, Conn., U.S.A.; Germiston virus (ts mutant 391) from Dr S. Ozden, Pasteur Institute, Paris, France; Lumbo virus (ts mutant 35) from Dr D. H. L. Bishop, University of Alabama, Birmingham, Ala., U.S.A.; Dugbe virus, Naples sandfly fever virus, Potpevi virus and Sicilian sandfly fever virus from Dr J. S. Porterfield, Sir William Dunn School of Pathology, Oxford, U.K.; and St Abb’s Head virus (M349) from Dr P. A. Nuttall, Institute of Virology, Oxford, U.K.

Batai, Bunyamwera, Clo Mor, Guaroa, Kairi, La Crosse, Maguari, Main Drain, Northway and Sathuperi viruses were obtained as lysates of suckling mouse brain and subsequently triple plaque-purified in BSC-1 cells. The stock of La Crosse virus used was an uncharacterized ts mutant obtained after 5-fluorouracil mutagenesis (C. R. Pringle, unpublished).

Cache Valley, Dugbe, Naples sandfly fever, Potpevi and Sicilian sandfly fever viruses were obtained as sucking mouse brain isolates and used directly without preliminary passage in tissue culture. Sicilian sandfly fever virus was subsequently passaged once in BSC-1 cells (see Results).

Germiston virus (ts mutant 391) (Ozden & Hannoun, 1980) and Lumbo virus (ts mutant 35) (Bishop, 1979) were obtained as tissue culture supernatant and passaged once in BSC-1 cells before use.

St Abb’s Head virus was obtained as an XTC-2 culture supernatant and subsequently triple plaque-purified in chick embryo cell monolayers. This virus was derived originally from a pool (M349) of L. uriae ticks which contained an orbivirus as well as the uukuvirus now named St Abb’s Head virus.

Preparation of virus stocks and assay by plaque titration. Virus stocks were prepared by infecting confluent monolayers of cells at low multiplicity (0-001 to 0-01 p.f.u./cell), and harvesting the culture fluid when the cells exhibited extensive c.p.e. or at 5 days post-infection if no c.p.e. was evident. The culture fluid was clarified by centrifugation, and aliquots of the clarified fluid were stored at −70 °C.

Plaque titration of Bunyaviridae in BSC-1 and XTC-2 cells followed the method of de Madrid & Porterfield (1969) using a carboxymethyl-cellulose overlay, as modified by Leake et al. (1977); titrations in CEF and BHK cells used an agarose overlay (Iroegbu & Pringle, 1981).

Radiolabelling of intracellular proteins. Cells in 30 mm Petri dishes were infected at a multiplicity of 1 to 5 p.f.u./cell. At various times after infection, the growth medium was replaced with 1 ml phosphate-buffered saline (PBS) containing either 30 μCi [35S]methionine (sp. act. 900 Ci/mmol) or 100 μCi [3H]mannose (sp. act. 16 Ci/mmol) and incubation continued at 31 °C for 1 h. The radioactive solution was then removed, the monolayers washed with cold PBS and the cells lysed in 200 μl protein dissociation mix (0-125 m-Tris–HCl pH 6-8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0-1% bromophenol blue). Cell lysates were boiled for 2 min and stored at −20 °C.

Radioimmunoprecipitation. Infected cells were labelled with [35S]methionine as described above, and after labelling, the cells were washed with cold PBS, scrapped into PBS and pelleted by centrifugation. The cell pellet was resuspended in 200 μl lysis buffer (0-15 m-NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0-1% SDS, 1 mM-phenylmethylsulphonyl fluoride, 0-01 m-Tris–HCl pH 7-4), incubated on ice for 30 min and vortexed for 10 s. Nuclei and other debris were removed by centrifugation for 5 min at 10000 g, and the lysate precleared by incubation with 50 μl 10% formalin-fixed Staphylococcus aureus (Immunoprecipitin, Bethesda Research Laboratories) for 30 min on ice. The bacteria were removed by centrifugation and 50 μl aliquots of the supernatant were reacted with either 10 μl normal serum, or 10 μl hyperimmune serum or 10 μl PBS, for at least 3 h on ice. One-hundred μl of Immunoprecipitin was added and incubation continued for 30 min. Immune complexes were collected by centrifugation for 20 s at 10000 g and washed three times with 500 mM-LiCl, 100 mM-Tris–HCl pH 8-5. The final pellet was resuspended in 50 μl of protein dissociation mix and boiled for 2 min.

Antiserum to purified Bunyamwera virus and St Abb’s Head virus were produced by intramuscular injection of rabbits with 100 μg of viral protein in complete Freund’s adjuvant at weekly intervals for 3 weeks followed by a boost 4 weeks later. Rabbits were bled 2 weeks after the final inoculation. Immune ascitic fluid from mice injected with Clo Mor virus was provided by Dr P. A. Nuttall.

Polycrylamide gel electrophoresis. Proteins were fractionated on polycrylamide gels in the presence of 0-1% SDS using the discontinuous buffer system of Laemmli (1970). The ratio of acrylamide to bisacrylamide was 75:1 and gels containing 12% or 15% acrylamide were used, as indicated in the figure legends. The molecular weights of virus-specified proteins were estimated by reference to a 14C-labelled standard protein mixture obtained from Amersham (catalogue number CFA.626).
RESULTS

Growth and plaque titration of Bunyaviridae in XTC-2 cells

The results of experiments to determine the ability of 18 members of the Bunyaviridae to produce plaques on XTC-2 cell monolayers are shown in Table 1; for reference other cell lines that we have observed to be permissive for these viruses are also listed. XTC-2 cells were infected with either suckling mouse brain homogenates or clarified supernatant fluids from infected tissue culture cells as indicated. It was found that all the representatives of the Bunyavirus, Nairovirus and Uukuvirus genera produced discernible plaques on XTC-2 cell monolayers within 4 to 5 days post-infection. Previously we reported (Watret et al., 1984) that neither of the phleboviruses used (Sicilian sandfly fever virus and Naples sandfly fever virus) produced plaques in the XTC-2 cell line. However, after passage of the mouse brain stock of Sicilian sandfly fever virus in BSC-1 cells, this virus did plaque in XTC-2 cells. On the other hand, the stock of Naples sandfly fever virus used did not plaque in XTC-2, BHK or BSC-1 cells.

The Xenopus line has some advantages over the other cell lines used: firstly, it has been our experience that the titres in XTC-2 cells are generally higher than in other cell lines, and secondly, plaques are discernible within 4 or 5 days for all tested viruses, whereas it takes 6 to 7 days to detect St Abb’s Head virus plaques in chick embryo fibroblasts and 11 to 12 days to detect Clo Mor virus plaques in BSC-1 cells.

Protein synthesis in infected XTC-2 cells

Bunyavirus genus

The identification of proteins induced in XTC-2 cells following infection with Bunyamwera virus was facilitated because of the inhibition of host cell protein synthesis. Seven infection-specific polypeptides were detected (Fig. 1): the structural proteins L, G1 and N, and four non-structural proteins (not found in purified virus preparations) of molecular weights 60000 (60K), 50K, 16K and 13K. It should be noted that in our hands the G2 protein of Bunyamwera virus is detected more variably and less reproducibly than has been reported by others (e.g. McPhee &

Table 1. Origin and host range of the Bunyaviridae used in this study

<table>
<thead>
<tr>
<th>Genus</th>
<th>Virus</th>
<th>Nature of inoculum for XTC-2 cells*</th>
<th>Plaque production in XTC-2 cells</th>
<th>Other permissive cell lines†</th>
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<tr>
<td>Bunyavirus</td>
<td>Batai</td>
<td>BHK</td>
<td>+</td>
<td>BHK, BSC-1</td>
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<td>+</td>
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<td>BSC-1</td>
<td>+</td>
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<td>+</td>
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<td></td>
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<td>SMB</td>
<td>+</td>
<td>BSC-1</td>
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<tr>
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<td>–</td>
<td>ND‡</td>
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<tr>
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<td>Potepli</td>
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<td>+</td>
<td>CEF</td>
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<td></td>
<td>St Abb’s Head (M349)</td>
<td>CEF</td>
<td>+</td>
<td>CEF</td>
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</table>

* SMB, Suckling mouse brain homogenate; BHK, BSC-1, CEF, clarified culture fluid from infected cells.
† This list is not exhaustive and only indicates alternative cell lines that we have shown to support the replication of these bunyaviruses.
‡ ND, Not done.
§ Supernatant fluid from BSC-1 cells infected with the SMB stock of Sicilian sandfly fever virus (see text).
Fig. 1. Bunyamwera virus-specific proteins in XTC-2 cells. Cells were labelled for 1 h with $[^{35}\text{S}]$methionine at the indicated times (h) post-infection. In this and other figures, MI indicates mock-infected cells. Radiolabelled proteins were separated on a 12% polyacrylamide gel.

Westaway, 1981; Short et al., 1982), but G2 can be detected if infected cells are labelled with $[^{35}\text{S}]$cysteine (R. M. Elliott, unpublished results). Three of the non-structural proteins we observed can be correlated with proteins reported by Short et al. (1982): the 13K and 16K proteins have direct equivalents, while the 60K protein may be equivalent to p50 of Short et al. (1982). However, we have no evidence which suggests that the 16K protein is a component of the virion, as reported by Short et al. (1982). We have detected the 50K protein, which is present in low amounts late in infection, only in Bunyamwera virus-infected XTC-2 cells, and it is possible that this is a virus-induced host protein.

XTC-2 cells were also infected with a number of other viruses belonging to the Bunyavirus genus. Representatives of three of the 13 serogroups of the genus were used: La Crosse virus and Lumbo virus of the California encephalitis serogroup, Sathuperi virus of the Simbu serogroup, and Kairi, Maguari, Main Drain, Northway, Batai, Cache Valley, Germiston and Guaroa from the Bunyamwera serogroup. Among the Bunyamwera serogroup viruses were two revertants of Maguari virus temperature-sensitive mutant 8 (Iroegbu & Pringle, 1981; Elliott et al., 1984) with atypical G1 proteins. The protein profiles of these are shown in Fig. 2. For most of these isolates the G1, N and the two smaller non-structural proteins were clearly detected.
Bunyavirus protein synthesis

Fig. 2. Proteins induced by members of the Bunyavirus genus in XTC-2 cells. Infected cells were labelled at 16 h post-infection for 1 h with [35S]methionine, and proteins fractionated on 15% gels. KAI, Kairi virus; MAG, Maguari virus and mutants; MD, Main Drain virus; NW, Northway virus; LAC, La Crosse virus; LUM, Lumbo virus; SAT, Sathuperi virus; BAT, Batai virus; CV, Cache Valley virus; GER, Germiston virus; GUA, Guaroa virus. NS indicates non-structural proteins analogous to the 16K and 13K proteins in Bunyamwera virus-infected cells.

Nairovirus genus

The nucleocapsid proteins of members of the Nairovirus genus are characteristically much larger than the corresponding proteins of other Bunyaviridae (Clerx & Bishop, 1981; Clerx et al., 1981). The major infection-specific protein detected in Clo Mor virus-infected XTC-2 cells had a mol. wt. of 50K (Fig. 3a) and has been designated the nucleocapsid protein. A minor protein of mol. wt. 45K was also detected late in infection. Since inhibition of host cell protein synthesis was poor, a combination of radiolabelling with [3H]mannose and immunoprecipitation (Fig. 3b, and c) was used, which confirmed that Clo Mor virus-specific proteins were analogous to those seen with other nairoviruses (Clerx et al., 1981). [3H]Mannose labelling of infected cells revealed a predominant glycoprotein (gp) of about 115K (Fig. 3b). Immunoprecipitation of infected cell extracts detected gp115 (weakly) and N, together with proteins of 90K and 80K (Fig. 3c); the 80K protein may correspond to G1 and the 115K and 90K proteins are probably analogous to the precursor glycoproteins reported by Clerx et al. (1981).

We have not been able to detect an equivalent of G2 in cell extracts reliably, but in pelleted supernatant fluid from infected cells the 45K protein was present in large amounts (Fig. 3d). Immunoprecipitation of [3H]mannose-labelled supernatant fluid from infected cells confirmed that the proteins designated gp90, gp80 and gp45 in Fig. 3(d) were indeed glycoproteins (data not shown). The gp45 protein would be somewhat larger than the molecular weights reported for
Fig. 3. Nairovirus-specific proteins induced in XTC-2 cells. (a) Time course of \(^{35}\text{S}\)methionine-labelled proteins in Clo Mor virus-infected cells. Cells were labelled for 1 h at the indicated times after infection, and proteins separated on a 12 % gel. (b) \(^{3}\text{H}\)Mannose-labelled Clo Mor virus-infected cells. Cells were labelled at 8 or 16 h after infection with a 1 h pulse; 12 % gel. (c) Immunoprecipitation of mock and Clo Mor virus-infected cells: c, cell lysate (PBS); pi, preimmune serum; i, immune serum; 12 % gel. (d) Virus-specific proteins in the pelleted supernatant from Clo Mor virus-infected XTC-2 cells labelled with \(^{35}\text{S}\)methionine; 15 % gel. (e) Comparison of the proteins induced in XTC-2 cells by Clo Mor (CM) virus and Dugbe (DUG) virus; 15 % gel.

the G2 proteins of other nairoviruses, which were in the range 30K to 40K (Clerx et al., 1981). However, these data show that Clo Mor virus shares structural characteristics with other nairoviruses. For comparison, the profile of XTC-2 cells infected with Dugbe virus is displayed in Fig. 3(e). Cells were infected with either Dugbe virus or Clo Mor virus and radiolabelled under similar conditions. Only the N protein (mol. wt. 49K) of Dugbe virus can be detected in infected cells. Both viruses showed a similar lack of inhibition of host cell protein synthesis; in this experiment the minor p45 protein could not be observed in Clo Mor virus-infected cells.

Phlebovirus genus

Fig. 4(a) shows the proteins detected in XTC-2 cells infected with Sicilian sandfly fever virus. A single infection-specific protein was observed in cell lysates: a 25K protein equivalent to the nucleocapsid protein. For comparison, the protein profile of infected BSC-1 cells is shown in Fig. 4(b). The inhibition of host cell protein synthesis was considerable compared to XTC-2 cells and in addition to the N protein, a protein of about 30K mol. wt. was detected. This second protein, which was present in large amounts in infected BSC-1 cells, may be analogous to the NS protein reported in cells infected with two other phleboviruses: Karimabad virus (Smith & Pifat, 1982) and Rift Valley fever virus (Struthers et al., 1984). It is possible that in Sicilian sandfly fever virus-infected XTC-2 cells the 30K protein is made in low amounts, and is masked by a co-migrating host protein(s). The difference in shut-off of host cell protein synthesis in the two cell lines is not understood, and was not due to differences in the multiplicity of infection used in the experiment.
Uukuvirus genus

St Abb's Head (M349) virus was suggested to be a member of the *Uukuvirus* genus on the basis of serological cross-reactivity with antiserum to Uukuniemi virus, the prototype of the genus (Nuttall *et al.*, 1981). Analysis of the proteins synthesized by St Abb's Head virus in XTC-2 cells (Fig. 5) would support this classification. St Abb's Head virus did not shut off host protein synthesis, but three proteins were detected in infected cell extracts: a 25K protein corresponding to the nucleocapsid protein and two minor proteins, p58 (58K) and p30 (30K), which are thought to be non-structural proteins (Fig. 5a, b). Detection of the non-structural proteins was dependent on the concentration of polyacrylamide in the resolving gel. A protein equivalent to p30 has been
reported in Uukuniemi virus-infected cells by Ulmanen et al. (1981). To detect other infection-specific proteins, immunoprecipitation experiments using a rabbit antiserum to St Abb's Head virus were performed. In addition to the N protein, a doublet corresponding to G1 and G2 proteins was detected in the immune complex (Fig. 5c). The mol. wt. of the G1/G2 doublet, 62K to 75K, is similar to the value reported for Uukuniemi virus (Ulmanen et al., 1981). The G1/G2 proteins were confirmed to be glycoproteins by labelling with [3H]mannose (not shown). Purified St Abb's Head virus (Fig. 5d) contained G1, G2 and N, but p58 and p30 were not detected. These results support the inclusion of St Abb's Head virus in the Uukuvirus genus.

Potepli virus, a strain of Uukuniemi virus (Kolman et al., 1966), showed a similar protein profile in infected XTC-2 cells (Fig. 5e). The N protein (mol. wt. 25K) was the major virus-specific polypeptide detected, and little shut-off of host cell protein synthesis was observed.

**DISCUSSION**

Our studies have shown that the XTC-2 cell line, derived from *Xenopus laevis*, is a suitable host to examine replication of viruses in the family Bunyaviridae. Using representative viruses from the four recognized genera, we have extended the observations of Leake et al. (1977), who first demonstrated the broad susceptibility of the XTC-2 cell line to infection by arboviruses. The XTC-2 cell line provides a useful tool to assay Bunyaviridae, and has the advantage that plaques are detected earlier for some viruses than in other susceptible cell lines. Furthermore, we have previously observed that Bunyamwera virus, St Abb's Head (M349) virus and Clo Mor virus display similar growth kinetics in XTC-2 cells and other susceptible cell lines, and that the yields are similar for the different virus/cell combinations (Watret et al., 1984). The phleboviruses were the least satisfactory in XTC-2 cells, though Sicilian sandfly virus did produce plaques, whereas Leake et al. (1977) reported that this virus caused a generalized cell death under the overlay. We were unable to demonstrate the recovery of Naples sandfly fever virus from suckling mouse
brain homogenates in any of the cell lines tested. Recently, Schmaljohn & Dalrymple (1983) have presented compelling evidence for the establishment of a fifth genus in the family Bunyaviridae, with Hantaan virus as the prototype. It will be of interest to determine whether Hantaan and related viruses will replicate in the XTC-2 cell line.

Radiolabelling of infected XTC-2 cells indicated that, in general, the patterns of protein synthesis were similar in the Xenopus and other susceptible cell lines. By using a common cell line (XTC-2) to examine the proteins of different viruses under standardized conditions, comparisons can be made which are unaffected by subtle changes caused by using different cell lines or by host-specific factors. However, this sometimes has the disadvantage that host cell protein synthesis is not markedly impaired compared to alternative cell lines (e.g. as with the phleboviruses) but this can be overcome by immunoprecipitation techniques.

A practical consequence of this aspect of the work was a biochemical characterization of two Scottish Bunyaviridae, Clo Mor virus (Main et al., 1976) and St Abb's Head (M349) virus (Nuttall et al., 1981). We have shown that the spectrum of proteins induced by Clo Mor virus resembles that of other members of the Nairovirus genus as described by Clerx & Bishop (1981) and Clerx et al. (1981). Thus, our data support the inclusion of Clo Mor virus in the Nairovirus genus, as suggested previously by Main et al. (1976) on the basis of complement fixation tests.

Nuttall et al. (1981) reported St Abb's Head (M349) virus to be antigenically related to Uukuniemi virus by complement fixation, and the data presented here indicate that this virus has a similar protein profile to that of Uukuniemi virus as described by Ulmanen et al. (1981). Thus, we confirm St Abb's Head (M349) virus to be a member of the Uukuvirus genus. Further analyses will be required to determine the degree of relatedness between St Abb's Head (M349) virus and Uukuniemi virus.

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