Defective RNAs in mosquito cells persistently infected with Bunyamwera virus

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Viral protein and RNA synthesis were compared in BHK and Aedes albopictus C6/36 (mosquito) cells infected with Bunyamwera virus. In BHK cells host protein synthesis was inhibited and viral proteins were detected until the cells died; in C6/36 cells there was little inhibition of host proteins and viral proteins could not be detected after 36 h post-infection. Relatively more S segment RNA than L or M segment RNA was produced in infected C6/36 cells compared to BHK cells. A persistent infection of C6/36 cells was established and the cells were passaged at weekly intervals for over a year. The titre of virus released from the cells and the level of viral RNA in the cells at different passages fluctuated markedly, but there was no simple relationship between virus titre and the amount of viral RNA. Northern blot analysis of viral RNA extracted from persistently infected cells revealed the presence of subgenomic RNAs derived from the L RNA segment. These defective RNAs were not packaged into nucleocapsids. The presence of the defective RNAs did not correlate with resistance of cells cloned from the persistently infected population to superinfection with homologous virus. Hence the role of these defective RNAs in the maintenance of the persistent state remains to be elucidated.

Introduction

Bunyamwera virus is the mosquito-borne prototype of the Bunyavirus genus in the family Bunyaviridae, which is a large group of mainly arthropod-transmitted viruses characterized by a tripartite negative-sense RNA genome. The genome segments are the L (large) RNA which encodes the L protein (RNA polymerase), the M (medium) RNA which encodes the two virion glycoproteins, G1 and G2, and a non-structural protein, NSm, and the S (small) RNA which encodes the nucleocapsid protein, N, and a second non-structural protein, NSs (reviewed by Elliott, 1990).

In common with other bunyaviruses such as La Crosse (Rossier et al., 1988) and Marituba viruses (Carvalho et al., 1986), and indeed arboviruses in general (Randolph & Hardy, 1988), Bunyamwera virus can replicate in both vertebrate and invertebrate cell cultures. However, the outcome of these infections is quite different: a lytic infection leading to cell death in mammalian cells, e.g. BHK cells, or a non-cytocidal, self-limiting infection leading to persistence in insect cells, e.g. Aedes albopictus mosquito cells (Kascak & Lyons, 1978; Newton et al., 1981). A previous report from this laboratory (Elliott & Wilkie, 1986) showed that in persistently infected A. albopictus C6/36 cells there was an over-representation of the S RNA segment. In addition, some features, such as fluctuating titres of released virus, suggested the presence of defective interfering (DI) RNAs (Holland, 1990), and subgenomic RNAs derived from the L segment were noted. In a subsequent detailed study of bunyavirus replication in mosquito cells, Rossier et al. (1988) suggested that the self-limiting nature of La Crosse virus infection of C6/36 cells is due to translational control by N. Later, Hacker et al. (1989) demonstrated that control is mediated by encapsidation of the S mRNA by N, thereby preventing its translation. No DI RNAs were detected in the persistently infected cells (Rossier et al., 1988). As this latter point is at variance with our previous observation, we have re-examined Bunyamwera virus persistence. During the early stages of persistence we found no gross differences between the behaviour of the two viruses; however, after long-term persistence a number of defective RNAs were detected in Bunyamwera virus-infected cells. The possible role of these defective RNAs in the maintenance of persistence is discussed.

Methods

Cells. A. albopictus C6/36 cells (Igarashi, 1978) were obtained from Dr D. L. Knudson (Yale Arbovirus Research Unit, New Haven, Conn., U.S.A.) and maintained at 28 °C in L-15 medium (Leibovitz, 1963) supplemented with 5% heat-inactivated foetal calf serum and
10\% tryptose phosphate broth. BHK-21 cells were maintained at 31 °C in Glasgow MEM containing 10\% calf serum and 10\% tryptose phosphate broth.

**Virus.** The Bunyamwera virus stock used in this study was plaque-purified three times in BHK-21 cells, and working stocks were grown in BHK-21 cells as described in Watret et al. (1985). Plaque titration was carried out as described by Iroegbu & Pringle (1981).

**Establishment of persistent infection.** A. albopictus C6/36 cells were grown to 90\% confluence and infected with 5 p.f.u. Bunyamwera virus per cell. After 4 days the cells were subcultured at a 1/10 split and thereafter at a 1/20 split at approximately 6 day intervals. The cells were maintained at 28 °C in supplemented L-15 medium.

**Analysis of viral proteins.** Radioabelling of cells with [\textsuperscript{35}S]methionine (Amersham) and preparation of cell lysates for SDS–PAGE have been described previously (Watret et al., 1985).

**Analysis of RNA.** Total cellular RNA was extracted by the acid-phenol–guanidinium thiocyanate protocol of Chomczynski & Sacchi (1987). Viral nucleocapsids were isolated from infected cells using the method described by Leppert et al. (1979) and Rossier et al. (1988). Cytoplasmic extracts from infected cells were centrifuged through a preformed 20 to 40\% CsCl gradient overlaid with sucrose. Bunyamwera virus nucleocapsids appeared as a sharp band in the centre of the gradient and the remaining infected cell RNA pellet. The pelleted RNA was used as the source of viral mRNA. The nucleocapsids were harvested using a syringe, diluted with NTE buffer (100 mM-NaCl, 10 mM-Tris–HCl pH 7.5, 1 mM-EDTA) and concentrated by centrifugation (Leppert et al., 1979). RNA was extracted from the pelleted nucleocapsids using the method of Chomczynski & Sacchi (1987).

**Slot blot analysis.** One-hundred µl 20 × SSC (3 M-NaCl and 0.3 M-sodium citrate) was added to each RNA sample (10 µl) and the RNA was blotted onto nitrocellulose (Schleicher & Schuell, 0.2 or 0.45 µm pore size) using a Schleicher & Schuell Minifold II. The nitrocellulose was then baked at 80 °C for 2 h.

**Northern blot analysis.** RNA was fractionated in 1 or 1.8\% agarose gels containing 5 mM-methylmercuric hydroxide for 5 h at 75 V with recirculation of the buffer (Perbal, 1988). The separated RNAs were transferred to nitrocellulose by capillary blotting in 20 × SSC, without pretreatment of the gel. Precipitation and hybridization were carried out at 42 °C for 4 h and 16 h respectively. Precipitation by sucrose with Multiprime (see below)-generated probes was in 20 ml of 5 × SSPE (0.9 M-NaCl, 50 mM-NaH2PO4, 5 mM-EDTA, pH 7-4), 50\% formaldehyde, 0.5\% low fat milk and 0.1 mg/ml carrier DNA. Hybridization was done in a similar buffer but with 0.1\% milk.

When riboprobes were used, hybridization was in 20 ml of 6 × SSPE, 50\% formaldehyde, 0.05 mg/ml carrier DNA, 0.05 mg/ml yeast tRNA, 0.1\% SDS and 5 × Denhardt’s solution, and hybridization was in the same buffer with 1 × Denhardt’s solution.

After hybridization the filters were washed twice (20 min each at room temperature) with 2 × SSC, 0.5\% SDS, and twice with 0.1 × SSC, 0.1\% SDS. When the actin probe (see below) was used, only the two high salt washes were done.

Multiprime (Amersham)-labelled probes were prepared from recombinant M13mp18 bacteriophages containing fragments of DNA complementary to the Bunyamwera virus S (Elliott, 1989a), M (Lees et al., 1986) or L (Elliott, 1989b) RNA segments using a kit from Amersham. The probes contained sequences complementary to nucleotides 5664 to 5864 of the L, 2233 to 2517 of the M and 550 to 952 of the S segment positive-sense RNAs.

Riboprobes were synthesized from full-length L, M and S RNA segment cdNA cloned under control of the bacteriophage T7 promoter (Jin & Elliott, 1991; unpublished data), using T7 RNA polymerase (Promega) and 50 µCi [\textsuperscript{32}P]CTP according to the manufacturer’s instructions.

The [\textsuperscript{32}P]-labelled actin probe was prepared from PstI-digested pMG1 DNA (a mouse cytoskeletal γ-actin cdNA cloned in pBR322; Peter et al. (1988)) using the Amersham Multiprime DNA labelling system.

**Results**

**Viral protein and RNA synthesis in infected cells**

The time course of viral protein synthesis over 72 h of infection of BHK and C6/36 cells is shown in Fig. 1. Cells were infected at the same multiplicity (5 p.f.u./cell) using the same stock of virus, and pulse-labelled with [\textsuperscript{35}S]methionine for 1 h at the times indicated. The protein patterns of the two infected cell types were strikingly different. In BHK cells there was a marked inhibition of host cell protein synthesis such that, by 20 h post-infection, the viral L, G1, N and NSs proteins (first seen 6 to 8 h post-infection) were virtually the only proteins detected. By 36 h post-infection essentially no proteins were detected during a 1 h pulse. The G2 and NSm proteins, which are not reliably detected (Elliott, 1985), were not identified in this experiment. In C6/36 cells there was little decrease in host protein synthesis, and only G1, N and NSs could be identified above the host protein background. G1 was detected between 16 and 36 h post-infection, N between 8 and 24 h post-infection (weakly at 36 h) and NSs between 12 and 24 h post-infection. No viral proteins were detected after 36 h post-infection, whereas host protein synthesis continued at a slightly reduced rate.

We next compared viral RNA synthesis in the two cell types by Northern blotting (Fig. 2). Total cellular RNA was fractionated on a methylmercuric hydroxide–agarose gel and hybridized with a mixture of [\textsuperscript{32}P]-labelled strand-specific cdNA probes to detect positive-sense RNA (Fig. 2a). Viral L, M and S RNAs were clearly seen in both cell types up to 72 h post-infection. The ratios of the three segments in the different cell types were different. In BHK cells there was apparently more L and M segment RNA relative to S segment RNA, whereas in mosquito cells relatively more S segment RNA than L and M segment RNA was detected. Fig. 2(b) shows a slot blot analysis specifically designed to detect S mRNA in the caesium chloride-pelleted RNA prepared from C6/36 cells, and demonstrates that S mRNA was abundant in the cells 48 h post-infection, after N synthesis could no longer be detected. Furthermore, total cellular RNA extracted from mosquito cells at times up to 72 h post-infection could be translated in vitro and the viral N protein was synthesized (data not shown), indicating that the S mRNA detected by slot blot analysis was intact and
Bunyamwera virus defective RNAs

Fig. 1. Protein synthesis in Bunyamwera virus-infected BHK (a) and A. albopictus C6/36 (b) cells. Cells were either mock-infected (lanes M) or infected with 5 p.f.u./cell Bunyamwera virus, and pulse-labelled for 1 h with $^{35}$Smethionine at 2, 4, 6, 8, 10, 12, 16, 20, 24, 36, 48 and 72 h (as indicated) post-infection. Cell lysates were fractionated on 12% polyacrylamide-SDS gels. Viral proteins L, G1, N and NSs are indicated.

Fig. 2. Positive-sense RNA synthesis in Bunyamwera virus-infected BHK and A. albopictus C6/36 cells. Mock-infected (lanes M) or Bunyamwera virus-infected cells were harvested at 6, 12, 24, 36, 48 or 72 h (as indicated) post-infection, and RNA was extracted. (a) Total RNA was extracted using guanidinium isothiocyanate, fractionated on a 1.0% agarose/methylmercuric hydroxide gel and blotted to a nitrocellulose filter. (b) Unencapsidated mRNA was prepared by pelleting a cytoplasmic cell extract throughout CsCl, and applied to a nitrocellulose filter by vacuum filtration. Encapsidated RNA was extracted from CsCl-banded nucleocapsids, fractionated on a 1.8% agarose gel and blotted to a nitrocellulose filter. Filters shown in (a) and (c) were hybridized with a mixture of Multiprime-labelled single-stranded cDNA probes (see Methods) which detect L, M and S positive-sense RNA as indicated. The filter in (b) was hybridized with the S segment positive-sense RNA-specific probe only.

functional. Analysis of positive-sense RNA packaged into nucleocapsids (Fig. 2c) again demonstrated the relative abundance of S segment RNA compared to L and M segment RNA in C6/36 cells compared to the ratio seen in BHK cells.

Viral RNA synthesis in persistently infected mosquito cells

Having established that Bunyamwera virus protein and RNA synthesis over the first 72 h of infection of C6/36
Fig. 3. Analysis of RNA levels in *A. albopictus* C6/36 cells persistently infected with Bunyamwera virus. Total cellular RNA was extracted at each passage of the persistently infected cells and aliquots were applied in quadruplicate to a nitrocellulose filter using a vacuum manifold. Strips of the filter were then hybridized with $^{32}$P-labelled riboprobes specific for each genome RNA segment (i.e. to detect negative-sense L, M or S RNA) of Bunyamwera virus, or with a $^{32}$P-labelled actin gene cDNA to estimate the relative amount of RNA applied to each sample. The titre of Bunyamwera virus released at the corresponding passage of the cells is shown below the slot blot filters.

Cells are comparable to those during La Crosse virus infection (Rossier *et al.*, 1988), we examined RNA levels in persistently infected cells by slot blot hybridization. A persistently infected cell line was established essentially as described previously (Elliott & Wilkie, 1986) and showed similar characteristics in terms of fluctuating titres of released virus at different passages (Fig. 3), the production of plaque morphology variants and resistance to superinfection with Bunyamwera virus (data not shown). Viral genome RNA in the cells at each passage was monitored by hybridization with segment-specific $^{32}$P-labelled riboprobes (Fig. 3). A replicate filter was hybridized with a $^{32}$P-labelled mouse actin gene cDNA which was washed at low stringency to give an indication of the amount of RNA applied in each sample. The level of viral RNA was normalized with respect to the actin signal by densitometry of the films, on the assumption that the actin mRNA level remained relatively constant at each passage. The hybridization results show (Fig. 3) that, not unexpectedly, the relative levels of viral RNA varied between different passages. More interesting, however, was the fact that the amount of individual segments appeared to vary independently, e.g. cells at passage 6 had more L RNA than M and S RNA, whereas at passage 13 both the L and S segments were considerably more abundant than the M segment.

The data shown in Fig. 3 did not reveal a simple correlation between the relative level of viral RNAs in the cells and the titres of virus released from the same cells.

We performed Northern blot analyses on RNA samples from selected passages of the persistently infected cells to obtain information on the sizes of the RNA species present (Fig. 4). Total cellular RNA was fractionated in a denaturing agarose gel and, following transfer to a nitrocellulose filter, the RNA was hybridized with segment-specific probes. Fig. 4(a) shows the result of sequential hybridization of the filters with first an L segment riboprobe and second an S segment riboprobe, in both cases detecting negative-sense RNA. Subgenomic L segment-derived RNAs were just visible at passages 2 and 6, and were clearly detected at passage 21 of the persistently infected cell line; a wider spectrum of defective L RNAs was seen in the sample from passage 58. The ratio of defective L segment RNA to full-length L RNA varied between different passages, and at passage 58 the defective RNA was much more abundant than the full-length L segment. No subgeno-
mic M segment RNA (data not shown) or S segment RNA was observed, but interestingly at passage 21 a more slowly migrating S segment RNA was detected. Fig. 4(b) shows defective L RNA detected at passage 13 of the persistently infected cells. The RNA in lane 1 was hybridized with an L segment-specific riboprobe which detects negative-sense RNA, whereas lane 2 shows another aliquot of the same RNA hybridized with a mixture of L, M and S segment cDNAs which detect positive-sense RNA. A different spectrum of defective RNAs was seen depending on the strand-specificity of the probe used for hybridization; the positive-sense RNAs observed may be only a subpopulation of those in the sample as the cDNA probe itself was not a full-length copy of the L segment (see Methods).

Previously we have reported that the S RNA segment is the major RNA species detected in virus particles released from cells persistently infected with Bunyamwera virus (Elliott & Wilkie, 1986). Here we investigated which RNAs were packaged into nucleocapsids by isolating intracellular nucleocapsids on caesium chloride gradients and analysing the extracted RNA by Northern blotting. An example is shown in Fig. 5 for nucleocapsids prepared at passage 18 of the persistently infected cells. When probed with a mixture of L, M and S segment riboprobes only negative-sense S segment RNA was readily detected. We also investigated whether the slower migrating S segment RNA detected in passage 21 cells (Fig. 4a) was packaged. Fig. 5 shows that this was not the case; only normal sized negative-sense RNA was contained in intracellular nucleocapsids. It is of note that the normal sized S RNA was much less abundant than the slower migrating S RNA (Fig. 4a) indicating strong selection for packaging the authentic sized S segment.

Viral RNA in cells cloned from the persistently infected cultures

The above experiments dealt with the population of persistently infected cells. To examine the characteristics of individual cells within the population, we cloned cells from four passages of the persistently infected cultures according to the procedure of Igarashi et al. (1977). Sixteen stable cell clones were established from each of passages 12, 20, 30 and 58, and were subcultured by shown to the right. The open squares indicate the more slowly migrating S RNA in cells at passage 21. (b) Analysis of total cellular RNA from cells at passage 13; RNA in lane 1 was probed with a 32P-labelled L segment riboprobe which detects negative-sense RNA, and that in lane 2 with a mixture of L, M and S segment 32P-labelled cDNAs which detect positive-sense RNAs. Full-length RNA species are indicated by arrowheads and defective L segment RNAs (def L) by square brackets.
standard procedures. Total cellular RNA was extracted from the clones and analysed by slot blot hybridization as described above. A wide variation in the level of viral RNA resident in the clones was observed, and some clones appeared not to contain any viral RNA (data not shown). The clones also showed a wide variation in their ability to resist superinfection with Bunyamwera virus, but there was no correlation between the amount of viral RNA in the cells and their superinfectibility (data not shown). Viral RNA in four clones derived from passage 20 was analysed by Northern blotting, and hybridized with the L segment riboprobe to detect negative-sense RNA (Fig. 6). Full-length L RNA could be detected in clones c5 and c8, but was barely detectable in c1 and could not be detected in c3. All clones contained a prominent defective L RNA species; the shorter RNAs in c1 and c3 had the same mobility on the gel, and were different from the RNAs in c5 and c8. Superinfection experiments (performed as described by Elliott & Wilkie, 1986) showed that c5 and c8 yielded less virus (3 × 10^4 p.f.u./ml and 1 × 10^4 p.f.u./ml respectively) than c1 (2 × 10^5 p.f.u./ml) and c3 (4 × 10^7 p.f.u./ml); the yield of virus from normal C6/36 cells was 1 × 10^6 p.f.u./ml. Hence the resistance of the persistently infected cells to superinfection with Bunyamwera virus appeared to correlate with the amount of full-length L RNA present, and not with the amount or occurrence of defective L RNA.

Discussion

In a previous study we noted the presence of subgenomic RNAs, derived from the L RNA segment, in A. albopictus C6/36 cells persistently infected with Bunyamwera virus (Elliott & Wilkie, 1986). In this paper we have described more detailed analyses of these RNAs, demonstrating that they are defective L RNA species. This point contrasts with the observations of Rossier et al. (1988) concerning La Crosse bunyavirus infection of mosquito cells, in which no defective RNAs were found.
It was important that we first showed the early events in Bunyamwera virus infection of C6/36 to be similar to those seen by Rossier et al. (1988) with La Crosse virus; Fig. 1 and 2 indicate that in gross terms the two systems are comparable. In addition, the newly initiated persistent infection by Bunyamwera virus mimicked that reported previously (Elliott & Wilkie, 1986), showing the reproducibility of the bunyavirus system. The most likely explanation for the failure of Rossier et al. to detect subgenomic RNA is that these authors looked at relatively early passages of their persistently infected cells (passages 4, 6 and 8), when the levels of defective RNAs are low; we would predict that if later passages were analysed, defective RNA would have been found.

The characteristics of the persistently infected cells (fluctuating titres of released virus and resistance to superinfection with homologous virus, Fig. 3; Newton et al., 1981; Elliott & Wilkie, 1986) are suggestive of the presence of DI genomes (Holland, 1990), but the absence of subgenomic RNA from the initial virus stocks suggests that DI RNAs are not involved in the establishment of the persistent infection (Rossier et al., 1988; Hacker et al., 1989). The defective L RNAs detected in cells persistently infected with Bunyamwera virus were not packaged into nucleocapsids or virions, and hence do not give rise to 'classic' DI particles. As reported previously, Bunyamwera virus DI particles only contain S RNA (Kascak & Lyons, 1978; Elliott & Wilkie, 1986), and the same most probably pertains for La Crosse virus (Rossier et al., 1988). The defective RNAs do not appear to be involved in resistance to superinfection either, as it was the amount of full-length L RNA rather than that of defective L RNA that correlated with superinfectibility (Fig. 6). Hence, strictly speaking, these RNAs are not DI RNAs but merely defective. Whether the defective RNAs play a role in the maintenance of persistence is not clear.

An intriguing observation was the presence of a larger S RNA in the persistently infected cells at passage 21 (Fig. 4a). Even though this larger RNA was more abundant than normal sized S RNA, the larger species was not encapsidated into nucleocapsids (Fig. 5). We are currently attempting to characterize the molecular structure of the larger S RNA.

Although the C6/36 line is a clonal isolate from a A. albopictus culture (Igarashi, 1978), preliminary analysis of cells cloned from the persistently infected cultures gave evidence that individual cells harboured different defective L RNAs and responded differently to superinfection with homologous virus. In addition, no Bunyamwera virus RNA was detected in some clones. Similar observations have been made with other persistent virus systems, e.g. BHK cells persistently infected with foot-and-mouth disease virus (de la Torre et al., 1989).

Therefore it will be of interest to examine these cells more closely to determine the cellular factors which may be involved in the maintenance of the persistent state.

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