Analysis of Viral and Defective-Interfering Nucleocapsids in Acute and Persistent Infection by Rhabdoviruses

By ELIZABETH A. GRABAU† AND JOHN J. HOLLAND*
Department of Biology, C-016 University of California, San Diego, La Jolla, California 92093, U.S.A.

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SUMMARY
We have isolated and characterized the RNA of intracellular virus nucleocapsids recovered from a number of cell cultures persistently infected with rabies virus or vesicular stomatitis virus (VSV). VSV persistent infections in BHK21, L cells and Aedes albopictus (mosquito) cells generally showed the presence of large amounts of defective-interfering (DI) nucleocapsid RNA and much smaller amounts of standard (B) nucleocapsid RNA. Persistent infections of BHK21 cells by two rabies virus strains, challenge virus standard (CVS-11) or HEP-Flury, were followed for several months during which time the ratio of DI to B nucleocapsid RNA cycled dramatically. We also observed coordinated fluctuations in the absolute amount of incorporation of $[^3\text{H}]$uridine into virus nucleocapsid RNA. Total incorporation was generally highest following a decrease in the relative amount of DI nucleocapsid RNA synthesis. At no time were DI nucleocapsids absent in any of the persistently infected cultures.

INTRODUCTION
Many different viruses are capable of establishing long-term persistent infections in vitro and in vivo (for review, see Stevens et al., 1978). Persistent infections by rhabdoviruses, a group of enveloped, negative-strand RNA viruses, are among the best-studied systems. Holland & Villarreal (1974) showed that vesicular stomatitis virus (VSV) readily established persistent infection of BHK21 cells but required the addition of homologous defective-interfering (DI) particles to do so. DI particles were necessary to attenuate the virulence of VSV for the host cell. These persistently infected cells continually shed variable, low levels of standard infectious virus (B virions) and DI virions into the culture medium. Further analysis of the cultures showed that DI particles were constantly present but that the populations of both standard and DI virus were continuously changing as determined by T1 oligonucleotide mapping (Holland et al., 1979).

Wiktor & Clark (1972) showed that rabies virus could also establish persistent infection of tissue culture cells. The ability of rabies virus to generate DI particles was demonstrated by Crick & Brown (1974). The rapid generation of DI particles and the slow growth of the virus give rise to persistent infection by rabies virus whether or not large numbers of DI particles are added to the inoculum (Holland et al., 1976b). Kawai et al. (1975) failed to detect any interferon in rabies virus persistent infection and were able to isolate DI particles from these cultures. They observed a cyclical production of both standard and DI virions by the persistently infected cells and concluded that DI particles play an important role in establishing and maintaining persistent infection by rabies virus. They also found a changing

† Present address: Department of Biology, University of Utah, Salt Lake City, Utah 84112, U.S.A.
population of virus in these persistent infections which was observed as a replacement of the original, wild-type, large plaque virus by a small plaque virus. Horodyski & Holland (1980) later demonstrated this same phenomenon for VSV persistent infection. Both groups found that, in addition to exhibiting the small plaque morphology, virus mutants arising during persistence were less susceptible to interference by the original DI particles that were used to establish the persistent infection (Kawai & Matsumoto, 1977).

The current study presents data on a number of long-term persistent infections by both VSV and rabies virus. With prolonged passage it has become increasingly difficult to detect any extracellular infectious virus released from these cultures although nearly 100% of the cells show virus antigen by fluorescent antibody staining and the cells remain resistant to homologous infection. In order to examine the virus present in these cultures we have analysed intracellular virus nucleocapsid species in infected cells (Roux & Waldvogel, 1981). We found DI nucleocapsids in all VSV- and rabies virus-infected cultures but the ratio of DI to standard RNA fluctuated markedly. We used this method to examine the early events leading to the establishment of VSV persistent infection. We confirmed the involvement of DI particles in persistent infection of BHK21 cells initiated by a plaque-purified VSV mutant that was isolated from another VSV culture (CAR4) after 69 months of persistence.

**METHODS**

**Cells.** BHK21 baby hamster kidney cells (American Type Culture Collection) were employed for virus production. Cells were grown in Eagle's minimal essential medium (MEM) containing 7% heat-inactivated calf serum. CER cells, which are hamster karyotype cells derived from a mixed culture of hamster and chicken cells by Dr T. Motokashi in Tokyo, were obtained from Dr J. Obijeski and were used for rabies plaque assays. L cells were obtained from American Type Culture Collection and *Aedes albopictus* cells were kindly provided by Dr M. Hewlett.

**Viruses.** The VSV strains were the wild-type Mudd-Summers Indiana strain (Mudd & Summers, 1970) or the tsG31 mutant of Pringle (1970). The rabies virus strains were the HEP-Flury strain from Dr H. Koprowski and the CVS-11 strain originally obtained from Dr G. Baer. Rabies virus propagation was as described by Sokol et al. (1968). Virus purification was carried out according to Holland & Villarreal (1975).

**Persistently infected cultures.** The persistently infected VSV culture, designated CAR4, has been described in detail (Holland & Villarreal, 1974; Holland et al., 1979) and has now been maintained for over 7 years in culture. The persistently infected rabies virus cultures were established by passage of the surviving cells from acute rabies infections (Holland et al., 1976b). We have a CVS-11 rabies persistent infection (CVSpi) that has now been passaged for over 4 years and the two HEP-Flury rabies persistent infections (HEP-1pi and HEP-2pi) that have been maintained for over 5 years and for over 8 months respectively.

**Labelling and isolation of intracellular nucleocapsid RNA.** Labelling of nucleocapsid RNA from both acute and persistent infection was essentially as described by Roux & Holland (1979). Monolayers were covered with 25 ml MEM plus 7% calf serum and 1.5 μg/ml actinomycin D (Calbiochem); 10 to 20 μCi/ml [3H]uridine (Amersham) was used for labelling. At the end of the labelling period (usually 24 h for persistently infected cultures) cells were collected and intracellular nucleocapsids were isolated according to the method of Roux & Waldvogel (1981).

**Analysis of virus nucleocapsids.** For the characterization of nucleocapsid RNA, nucleocapsids were resuspended in TEN (50 mm-tris-HCl pH 7.6, 0.5 mm-EDTA, 0.1 μm-NaCl) plus 0.5% SDS and centrifuged on 5 to 23% sucrose gradients. For analysis of virus proteins, purified virus or virus nucleocapsids were resuspended in loading buffer and electrophoresed in 10% polyacrylamide gels according to the method of Laemmli (1970).
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Fig. 1. Sucrose gradient profiles of VSV nucleocapsid RNA. RNA from isolated nucleocapsids was centrifuged on 5 to 23% sucrose gradients in an SW50.1 rotor for 110 min at 45000 rev/min. Fractions were collected from the bottom of the centrifuge tubes directly into scintillation vials. 

(a) Extracellular VSV B virions labelled with $[^3H]$uridine were purified from culture medium after 20 h of acute infection, disrupted with Nonidet P40 and subjected to the intracellular nucleocapsid isolation procedure (see Methods). The nucleocapsids banded at exactly the same density in CsCl as intracellular nucleocapsids from infected cells. 

(b) $[^3H]$uridine-labelled RNA from VSV DI 0-22 was extracted from purified DI particles and centrifuged on 5 to 23% sucrose gradients as a size marker. 

(c) BHK21 cells were infected with a mixed population of B and DI virus; intracellular nucleocapsid RNA was centrifuged on sucrose gradients. 

(d) Cells were infected as in (c) but the input ratio of DI to B virions was five times greater. Nucleocapsid RNA profiles on sucrose gradients reflect this difference.

**RESULTS**

*Intracellular virus nucleocapsid RNA from acutely and persistently infected cell cultures*

Villarreal & Holland (1976) studied virus-induced intracellular RNA synthesis in both VSV and rabies virus persistent infection. The RNA species present in VSV persistent infection were similar to those from acute infection with two notable differences. B virion-sized 42S RNA was seldom synthesized in persistently infected cells (except in rare cases of cytopathology) and the synthesis of an additional RNA of the DI size class was observed. RNA synthesis by HEP-Flury virus was more difficult to study because labelled RNA could not always be analysed quantitatively due to aggregation and to failure to migrate beyond the origin of the formamide-urea gels used for characterization. When analysis was possible, a complex pattern of rabies RNA synthesis was observed, presumably due to the rapid generation of multiple DI species by rabies virus. Similarly to VSV, persistent infections showed a lack of B virion-sized RNA synthesis during the 1.5 h labelling period.

In the present study we isolated intracellular virus nucleocapsids on CsCl gradients prior to characterization of the RNA in order to eliminate the large excess of virus mRNA present in infected cells. To determine whether the technique detected the presence of both B and DI nucleocapsids and quantified variation in their relative amounts, the isolation procedure was tested on acute VSV infection with a mixed B and DI particle inoculum. Fig. 1 shows the results for two such mixed infections starting with differing input ratios of B and DI particles (Fig. 1c, d). With increased DI particle input, the relative amount of DI to B RNA in nucleocapsids increased as would be expected. Control nucleocapsids isolated from purified...
VSV B virions alone and from a DI 0.22 particle were included for size comparison (Fig. 1 a, b). Fig. 2 shows the sucrose gradient profiles of intracellular nucleocapsid RNA from several other acute infections. It should be noted that early in VSV infection (5 h), while there is a high level of ongoing replication, a trailing shoulder was observed extending from the 42S B peak (Fig. 2 b). By 9 h, when a greater percentage of total label is already in completed 42S virus RNA, this shoulder (presumably of replicative intermediates) disappeared (Fig. 2a). This shoulder is not mRNA because the CsCl isolation procedure frees and pellets essentially all mRNA away from the nucleocapsids and cannot be mistaken for DI RNA (compare Fig. 1). Similar profiles were observed for VSV B acute infections of A. albopictus cells (Fig. 2c) and for acute HEP-Flury and CVS-11 rabies infections of BHK21 cells (Fig. 2d, e respectively). These rabies virus stocks are relatively free of DI particles and an additional high m.o.i. passage is required before DI particles appear.

The profiles of nucleocapsid RNA from VSV persistent infection of a number of different cell lines are shown in Fig. 3. Persistent infections of BHK21, L cells and A. albopictus cells showed many similar characteristics. DI nucleocapsid RNA was always present and the relative amount of B nucleocapsid RNA varied from barely detectable (if at all) as was usually the case for CAR4 (Fig. 3 a) to significant levels (Fig. 3 b), but in all cases the ratio of DI genomes to standard virus was very high. Fig. 3 (c, d) shows persistently infected A. albopictus cultures at two different times, illustrating the variable amount of B nucleocapsid RNA. For any given time in the cases that we tested, DI to B RNA ratios were unaffected by temperature (22 °C, 33 °C, 37 °C) or confluency of the monolayers (data not shown). All of these results confirm the involvement of DI particles in persistent infection in vitro.
Fig. 3. Sucrose gradient profiles of isolated nucleocapsid RNA from VSV persistent infections. All persistently infected cultures were labelled for 24 h with 20 μCi/ml [3H]uridine in the presence of 1.5 μg/ml actinomycin D. Sucrose gradient analyses were performed as in Fig. 1. (a) Nucleocapsid RNA from CAR4 at 88 months of persistence. (b) Nucleocapsid RNA from L cells persistently infected by VSV tsG31. (c) Nucleocapsid RNA from A. albopictus cells persistently infected by VSV CAR4 5 year virus. (d) Nucleocapsid RNA from the same culture as in (c), approx. 2 weeks later.

Rowlands et al. (1980) reported the isolation of a VSV mutant from CAR4 after 69 months of persistent infection that was able to establish persistence without the addition of DI particles. Although infection was initiated with a DI-free clone of virus, by the time the culture had recovered from initial cytopathology, DI particles were being shed into the medium, and they may have arisen early during slow replication of the mutant. To study in greater detail the early events occurring during establishment of persistent infection by this mutant, we followed intracellular nucleocapsid RNA synthesis in these cells. Plaque-purified 69 month CAR4 virus was used to infect several BHK21 monolayers. Intracellular nucleocapsid analyses were performed at intervals on these cultures. Up to the time they exhibited severe cytopathology (about 2 weeks) no significant DI nucleocapsid RNA synthesis was observed and, at that point, culture medium was used to infect new BHK21 monolayers in order to further characterize intracellular nucleocapsids. The first analysis after the onset of cytopathology showed almost exclusive synthesis of DI nucleocapsids, suggesting that low levels of DI particles were generated earlier.

Characterization of intracellular nucleocapsids from two rabies virus persistent infections

Kawai et al. (1975) showed the cyclical nature of rabies B and DI virus production in persistent infections initiated in BHK21 cells. They followed these cultures for up to 45 passages after initial infection during which time mature virus and DI particles released into the culture medium cycled regularly at approx. 2-week intervals. We present below an analysis of nucleocapsids recovered from cultures persistently infected with either HEP-Flury or CVS-11 rabies virus. There was very little infectious virus being shed by these cultures at any time. Recent assays for the presence of infectious virus over a 3-week period detected it
only once in the most recently established persistent infection (titre $10^3$ p.f.u./ml) and not at all in the other cultures.

Therefore, in order to follow virus synthesis by these cultures it was necessary to examine the intracellular nucleocapsids. Fig. 4 and 5 show representative sucrose gradient profiles of nucleocapsid RNA from two of the rabies persistently infected cultures, CVSpi and HEP-2pi, at various intervals. The cyclical variation (with a 2 to 3 week period) in the relative amount of B and DI nucleocapsid RNA was clearly demonstrated in both cases. Profiles were similar for a third rabies persistent infection (HEP-1pi) but data are not presented because the total $[^3H]uridine$ incorporation was so low that at times it was not significant. At no time were DI nucleocapsids absent from these cultures but B nucleocapsid RNA synthesis was absent or barely detectable at several points (see Fig. 4, day 1, 27, 53; Fig. 5, day 3, 27, 42). Note also in Fig. 5 the change with time in the DI population as evidenced by a size shift of the DI RNA peak and its resolution into two separate size classes (Fig. 5, day 27, 42, 58). This is not
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Fig. 5. Sucrose gradient profiles of nucleocapsid RNA from HEP Flury rabies persistently infected cells. Labelling was performed as in Fig. 3 and sucrose gradients were centrifuged as in Fig. 1. This persistent infection was analysed at frequent intervals from 5 to 7 months of persistence.

Fig. 6 shows the proteins of all three rabies virus persistent infections (lanes b to d) as compared to total protein from purified virions of both VSV and rabies virus. The major protein observed in the nucleocapsids from these persistent infections is the rabies N protein. The nucleocapsids from CAR4 (lane f) likewise verify the presence of VSV N protein. T1 oligonucleotide mapping of virus RNA obtained from HEP-2pi also verifies that it is rabies virus RNA (data not shown).

The cycling of DI to B nucleocapsid RNA in rabies persistent infection is shown in Fig. 7. The DI/B RNA ratios were calculated using the relative amounts of incorporation into DI and B peaks from the sucrose gradient profiles. We chose a conservative estimate of the size of DI RNA (half genome size) to convert to a particle ratio. Since DI RNA species are usually smaller in size, the excess number of DI nucleocapsids for each point is probably even surprising in light of studies on the changing B and DI populations in VSV persistent infection (Holland et al., 1979).

To verify that the isolated nucleocapsids were of rabies virus origin we examined their proteins by polyacrylamide gel electrophoresis. The major protein observed in the nucleocapsids from these persistent infections is the rabies N protein. The nucleocapsids from CAR4 (lane f) likewise verify the presence of VSV N protein. T1 oligonucleotide mapping of virus RNA obtained from HEP-2pi also verifies that it is rabies virus RNA (data not shown).
Fig. 6. Polyacrylamide gel analysis of rabies and VSV proteins. Virus proteins from isolated nucleocapsids or purified virions were electrophoresed in 10% gels according to the method of Laemmli (1970). Assignment of rabies virus proteins was according to Kawai (1977) and Naito & Matsumoto (1978). Protein bands were visualized by staining with Coomassie Brilliant Blue. (a, g) Proteins from purified Mudd-Summers B VSV. (b) Proteins from isolated nucleocapsids from HEP-2pi. (c) Proteins from isolated nucleocapsids of HEP-1pi. (d) Proteins from isolated nucleocapsids from CVSpi. (e) Proteins from purified CVS-11 B virions. (f) Proteins from purified HEP-Flury B virions. (h) Proteins from purified 87 month CAR4 B virions. (i) Proteins from purified VSV tsG31 B virions. (j) Proteins from isolated nucleocapsids from CAR4 after 91 months of persistence. The observed slight differences in mobilities of virus proteins are due to strain differences and to mutations accumulating during long-term persistence.

greater than presented here. This fluctuation is analogous at the intracellular level to that observed by Palma & Huang (1974) for VSV and by Kawai et al. (1975) for rabies virus, where the relative concentrations of extracellular B and DI particles were observed to cycle in an out-of-phase manner due to the inhibition of virus by DI particles coupled with dependence upon helper virus. The total amount of actinomycin D-resistant incorporation of [3H]uridine into rabies nucleocapsid RNA (both B and DI) is also plotted in Fig. 7. Unlike the DI/B RNA ratio, which is unaffected by the confluency of the monolayers at the time of labelling, total incorporation might vary depending upon how many cells were present. To control for this we always chose cell monolayers just as they reached confluency (approx. 10^7 cells). The fluctuation of total incorporation showed a correlation with the relative ratio of DI/B nucleocapsid RNA synthesized in these cultures. The amount of incorporation was highest following a decrease in the DI/B ratio. This indicates that the presence of a large excess of DI nucleocapsids greatly decreases virus replication as might be expected from replicative interference (Perrault & Holland, 1972; Huang & Manders, 1972; Khan & Lazzarini, 1977).

**DISCUSSION**

During each passing year of persistent infection, we have observed increasingly lower levels of extracellular virus being released by carrier cells. Analysis of intracellular nucleocapsid RNA provides the advantage of detecting synthesis and accumulation of genomes that may not be reflected in the virus species released from infected cells. All the persistently infected cultures we have examined share in common the constant presence of DI nucleocapsids.
Intracellular DI genome synthesis always exceeds the amount of standard genome synthesis at any given time in persistent cultures, although the relative ratios vary considerably with time. We have observed the presence of DI nucleocapsids in VSV persistent infection of several cell lines, including BHK21, L cells and A. albopictus cells and have shown that the relative ratios of standard to DI nucleocapsid RNA synthesis are unaffected by temperature. We have followed the early events involved in establishing VSV persistence with a slowly replicating ts (76 month) mutant and have seen the onset of DI genome synthesis occurring in cells at the point where slow but acute cytopathology proceeds to non-cytolytic persistence. Together these results again suggest that the ability of virus to generate and replicate DI particles is at least in part responsible for the establishment and maintenance of persistence.

Other mechanisms that have been invoked to explain persistence include temperature-sensitive mutants (Fields & Raine, 1974; Preble & Youngner, 1973; Youngner et al., 1976), the action of interferon (Ramseur & Friedman, 1977), extensive multiple mutations of virus genomes (Holland et al., 1980) or a combination of factors (Nishiyama, 1977; Nishiyama et al., 1978). Youngner et al. (1976) found no mature DI particles in their L cell cultures persistently infected with VSV and implicated ts mutants in the establishment and maintenance of persistence. In our VSV persistent L cell culture, intracellular DI nucleocapsids were observed both at 33 °C and at 37 °C. This provides further evidence that
a number of different mechanisms and not just a single phenomenon may be involved in RNA virus persistence.

The detection of intracellular DI nucleocapsids during in vitro rabies virus persistence is intriguing because of possible parallels to natural infection, and to abortive infection (Wiktor et al., 1972; Lodmell et al., 1969). Huang & Baltimore (1970) originally suggested that DI particles may be important determinants of virus persistence and may influence the eventual outcome of acute virus diseases. The possible role of DI particles in the pathogenesis of rabies infections remains uncertain (Holland & Villarreal, 1975; Clark & Ohtani, 1976; Wiktor et al., 1977; Wunner & Clark, 1980). Infection by rabies virus in nature shows a variable but sometimes very long incubation period compared to the closely related rhabdovirus, VSV. Rabies is most often transmitted from one host to another by bite, and the virus then spreads via the nervous system until finally reaching the brain and eliciting typical disease symptoms. In order to ensure spread of the disease, this insidious virus must not kill its host before it has first altered the behaviour of the infected animal while causing shedding of virus in the saliva. In natural infection by rabies virus, DI particles could be in part responsible for its ability to persist for long periods in the nervous system. The ability of rabies virus to generate DI particles has been known for some time (Crick & Brown, 1974) and persistent infection by rabies virus in vitro has been demonstrated by several groups (Wiktor & Clark, 1972; Kawai et al., 1975). Holland et al. (1976b) showed that persistent infection can be established by a plaque-purified clone of rabies virus which rapidly generated multiple DI species. DI particles can be generated in vivo in newborn mice (Holland & Villarreal, 1975) or in vitro by numerous fixed virus strains (Crick & Brown, 1974; Holland et al., 1976a; Clark et al., 1981). Clark and co-workers found that various fixed virus strains differed in their pathogenic potential for mice but they found no apparent correlation between this potential and the presence of DI particles in the inoculum. They did not examine the possibility that DI particles may be generated de novo in the nervous system. Possible evidence for the presence of DI particles in the brains of infected animals is the electron microscope observation of brain-associated particles in numbers that far exceed the infectious virus titres obtained from these infected brains (Murphy, 1977). We are initiating experiments to analyse the RNA of nucleocapsids from the brains of animals infected with fixed and field strains of rabies virus.

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