JOURNAL OF VIROLOGY, March, 1976, p. 805-815

Vol. 17, No. 3
Printed in U.S.A.

Factors Involved in the Generation and Replication of Rhabdovirus Defective T Particles

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Received for publication 22 October 1975

Previous indications that cloned B virions might be genetically predisposed to generate a particular defective T particle are shown to be inaccurate. T particle generation was found to be a much more random process than was previously believed. We show that the previously observed generation of particular sizes of T particles by B virion pools is due to the random generation of T particles during preparation of first-passage pools of cloned B virions, and these breed true during the additional passages needed to produce visible quantities of T particles. It is also shown that different host cell lines selectively amplify different T particles, suggesting a strong role of host cell factors in T particle replication. Surprisingly, our line of HeLa cells did not generate or replicate detectable T particles of vesicular stomatitis virus (VSV) Indiana after either serial undiluted passage or direct addition of T particles, even though the added T particles strongly interfered with B virion replication. In contrast to VSV, rabies virus generates large amounts of T particles during the first passage of cloned B virions, and every rabies-infected baby hamster kidney-21 cell culture evolves into a persistent carrier state. We find that T particle RNA is biologically inactive although T particle nucleocapsid ribonucleoprotein replicates and interferes in cells coinfected with B virions. Efforts to study the mechanism of T particle generation by in vitro attempts to generate T particles or modify their size (using sheared ribonucleoprotein or chemical or UV mutagenesis) were unsuccessful. The kinetics of UV and nitrous acid inactivation of T particles indicate a smaller target size relative to B virions, even after correcting for lengths of RNA molecules. The intercalating dye proflavine does not photosensitize VSV B virions or T particles when present during replication, indicating that there is little or no RNA base pairing in the helical nucleocapsids of either.

After Cooper and Bellett (5) found a transmissible interfering particle of vesicular stomatitis virus (VSV), Huang and Wagner (15, 16) and Hackett (10) showed that the phenomenon was due to particles of truncated length containing subgenomic lengths of RNA. These truncated T particles are defective (due to the deleted portions of the genome) and hence unable to replicate in the absence of infectious B virions (15, 16). They are apparently genetically inert and unable to code for any viral protein because they lack virion transcriptase activity (8, 14, 20, 21, 23). This is probably due to the unsuitability of the T particle RNA as a transcription template since Emerson and Wagner showed the presence of enzyme in T particles (9).

Because of their smaller size, T particles can be purified away from B virions (15, 16), even completely freed of them (8), but B virions free of T particles can be obtained only by cloning, preferably serial cloning as described by Stampfer et al. (29). The mechanism of generation of these deletion mutant T particles is not known, but Reichmann et al. (22) showed that different cloned mutant B virion pools each regularly generated a unique T particle upon serial high-multiplicity passage. Perrault and Holland (20) observed that different cloned B virion pools derived from the same wild-type stock pool of VSV New Jersey each regularly generated the same T particle sizes (but different sizes of T particle were produced from each cloned pool). This invariable production of the same size of T particle upon three or more serial undiluted passages of a clonal stock of mutant or "wild-type" B virions suggests that the kind of T particle generated might be

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genetically predetermined by each clone of B virion. However, we recently showed that the maximum amplification factor for T particle replication during a single passage in the presence of high multiplicities of B virions is 20,000-fold (12a). Therefore, it can be calculated that B virion pools generated by a single passage of virus picked from a clone probably already contain seed quantities of T particles to generate visible bands of T particles after three additional undiluted passages. We show below that the regular production of unique T particles by clonal pools of B virions is in fact due to the presence in these pools of seed amounts of T particles generated during the preparation of the pool of a single passage of the virus picked directly from the plaque. It is shown that virus picked directly from plaques of mutant or wild-type VSV (Indiana) contains no T particles, but generates them in a rather random and unpredictable fashion during preparation of a first-passage pool. It is also shown that different host cells tend to select for certain T particles over others, and that cloned rabies virus, unlike VSV, generates visible amounts of T particles during the first passage of a B virion pool (probably because they are generated during the 7 or more days it takes to form a plaque).

Choppin (3) with influenza virus, Huang and Baltimore (13), Coward et al. (6), and Perrault and Holland (19) with VSV, and Portner and Kingsbury (21) with Sendai virus have all observed a strong influence of host cell type on defective interfering (DI) particle interference. We show a remarkable inability of our HeLa cell line to replicate VSV Indiana T particles or to produce them in an indefinite number of passages.

Brown et al. (2) have shown that VSV B virion nucleocapsid is infectious. However, VSV RNA is not infectious, nor would it be expected to be since this negative-strand virus has been shown by Baltimore et al. to carry a transcriptase that must produce positive-strand mRNA before viral protein can be produced to replicate and encapsidate viral RNA (1). Sreevalsan (28) has reported that T particle RNA does interfere with B virion replication. This is not unexpected since B virions provide all viral proteins necessary to replicate and encapsidate T particle RNA. However, we show that T particle RNA is biologically inert in our hands. It was found to neither interfere nor replicate more T particles in cells coinfected with B virions (even employing an extremely sensitive amplification test). T particle nucleocapsid is shown to replicate and to interfere after facilitated uptake into cells.

Almost nothing is known about mechanisms of DI particle generation with any RNA virus except that they are formed upon serial high-multiplicity passage as von Magnus first reported (30). We show below using B virion and T particle ribonucleoprotein (RNP), ultrasonic shearing, and chemical mutagenesis that trivial breakage mechanisms probably are insufficient to generate T particles.

MATERIALS AND METHODS

Cells and virus. Except where otherwise specified, baby hamster kidney-21 (BHK-21) cells were employed for all virus assays and virus production. Both BHK-21 cells and HeLa cells were originally obtained from Flow Laboratories and were cultivated in Eagle minimal essential medium (MEM) containing 7% heat-inactivated calf serum. The L cell mouse fibroblast line, PK_{15} pig kidney line, and RK_{13} rabbit kidney line were obtained from the American Type Culture Collection. The NHP line is a primary human fibroblast line established in this laboratory, and the VA_2 line of simian virus 40-transformed human fibroblasts was obtained from Gordon Soto. VSV was the Indiana serotype and it was grown and purified as described elsewhere (12a). Rabies virus was the high egg passage (HEP) Flury strain and it was kindly provided by Hilary Koprowski of the Wistar Institute as a pool that had been cloned three times on BHK-21 cells. It was cloned once more in this laboratory, and then a pool was prepared by inoculation of BHK-21 cells at a multiplicity of infection (MOI) of 0.001. This pool had a titer of 7×10^8 PFU/ml and was stored frozen at −70 C in small aliquots until used. Plaque assays for rabies virus were as described by Sokol et al. (27) with agarose-suspended BHK-21 cells in MEM without calf serum but with 0.4% bovine serum albumin instead. The infectious B virions of rabies employed here were all from this pool. The infectious B virions of VSV Indiana employed here were all from a first-passage pool prepared by infection of BHK-21 cells at a low MOI with virus picked directly from an isolated plaque that was itself produced by five successive plaque isolations without intervening passage in liquid medium. VSV plaque assays were carried out on monolayers of BHK-21 of cells under 0.4% agarose. Sterile Pasteur pipettes were employed to recover virus from well-isolated plaques without any cell staining. For visualization and counting of plaques in virus assays, agarose medium was poured off the monolayer, and the cells were stained with crystal violet as described previously (11).

Amplification test for T particles. This method for quantitation of small numbers of T particles has been described in detail elsewhere (12a). Briefly, it involves adsorption of 1 ml of T particles (or medium to be assayed for T particles) to monolayers of 2×10^7 BHK-21 cells for 2 h at 37 C. Cloned B virions were also added at a MOI of 100 to ensure that all cells were infected with virus to support T particle replication, and to ensure that only a single cycle of replication occurred. After 20 h of incubation at 37 C,

virus yields were purified by differential centrifugation and sucrose gradient centrifugation in 5 to 40% sucrose gradients containing TEN buffer (8) for 30 min at 35,000 rpm to display arrays of B virions and T particles. It has been shown elsewhere (12a) that in this procedure a limiting number of T particles (MOI of approximately 1.0 or less) are amplified approximately 10^4-fold in the case of VSV (maximum, 20,000-fold) and slightly less in the case of rabies (maximum, 5,000-fold). T particle quantitation is carried out by Lowry protein determinations on purified T particles assuming a particle weight of 1.5×10^8 daltons for B virions and a protein content of approximately 70% for B virions and T particles (31). Whenever T particles were present in very small amounts, several monolayers were employed for amplification, or (where indicated) two amplification tests were carried out in series (over 10^8-fold amplification factor) (12a).

Chemicals. Proflavine was obtained from National Aniline, cordycepin was purchased from Sigma, 8-azaguanine was obtained from Nutritional Biochemical Co., 5-fluorouracil was purchased from Hoffman La Roche, and rifampin was a gift from E. P. Geiduschek of this department.

RESULTS

Consistent generation of VSV T particles by cloned B virion pools. Reichmann et al. (22) demonstrated clearly that cloned seed stocks of various temperature-sensitive (*ts*) mutants of VSV consistently generate the same size of T particles upon serial high-multiplicity passage of the same cloned mutant pool, but that each mutant pool generates its own characteristic size of T particles. Schincariol and Howatson (26) also observed consistent production of a long T particle by a heat-resistant mutant, and Perrault and Holland (20) observed generation of differing sizes of T particles by different clonal isolates of VSV New Jersey. The data on the right hand side of Fig. 1 demonstrate that a clonal seed pool of the *ts* 31 mutant of Pringle (kindly provided by E. Reichmann) repeatedly generates the same very short T particle (one-fifth genome size) during three independent passage series of three passages, each in BHK-21 cells. This confirms the data of Reichmann et al. (22). Fig. 1 also shows that a clonal stock of wild-type VSV Indiana (prepared after six consecutive clonal isolations of B virions) regularly generates the same two moderate-sized T particles during six independent passage series (of three serial undiluted passages each), and approximately the same ratios of each T particle were produced after each passage series. Surprisingly, the fifth clonal isolate (from which the previously mentioned sixth clonal isolate was prepared) regularly generates a single T particle upon three serial undiluted passages

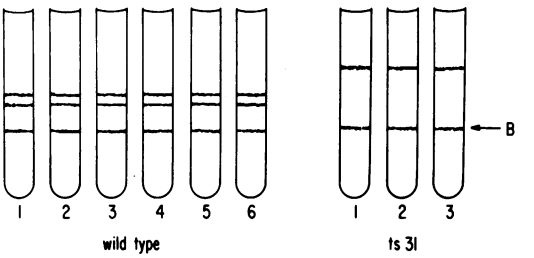

FIG. 1. *Consistent production of identical T particle types after independent serial undiluted passage series of wild-type VSV or ts 31 clonal pools. First-passage pools of B virions prepared from plaque isolates of wild-type VSV Indiana and the ts31 mutant (22) were each passaged three times serially with adsorption of 2 ml of the yield from each previous passage onto 2×10^7 BHK-21 cells. The third-passage virus yields from each separate passage series were purified from the culture medium after 20 h of incubation by differential centrifugation (8), suspension of viral pellets in TEN buffer, mild sonication to disperse aggregates, layering on top of 5 to 40% sucrose gradients in TEN buffer and centrifugation for 30 min at 35,000 rpm in the SW41 rotor in a Spinco ultracentrifuge at 4 C (8). The B virion and T particle bands were visualized in the dark with obliquely transmitted light. The position of each band was marked on each tube with a marker pen, the tube was emptied and sterilized with detergent-sodium hypochlorite solution, and the exact position of each band was traced onto paper at the ink mark positions on the cleaned, dry tubes. The amounts of virus protein present in the various bands of one of the six wild-type sucrose gradient analyses were as follows: B virion band, 207 μg; lower T particle band, 47 μg; upper T particle band, 63 μg. Very similar concentrations of each of these were seen in the other five gradients of the wild-type passage series.*

(data not shown). This invariable production of certain T particle types by clonal pools of B virions might be explained as being a genetic characteristic of each cloned B virion pool (with each wild-type or mutant clone carrying a mutation that determines the kind of T particle[s] that will be generated upon serial undiluted passages). Alternatively, it could merely be due to the presence in each clonal B virion pool of T particles already generated in small numbers during preparation of the B virion stock pool (during a single passage at low MOI of the clonal isolate). These two alternatives are tested below.

Nonuniform generation of T particle sizes during independent serial undiluted passages of B virions picked directly from isolated plaques. If the basis for the uniform production of certain T particle sizes (Fig. 1) is the presence of T particles in each first-passage pool of B virions, then this might be avoided by starting independent passage series with the B

virions picked directly from a plaque. Figure 2 shows that this is the case, since serial undiluted passage series from the sixth clonal isolate B virions did not consistently generate the same size T particles. Instead, arrays of different-sized T particles were observed, and these were different for each independent passage series from this clonal B virion isolate. Clearly, the types of T particle produced are not entirely genetically predetermined by the B virion. The sole difference between this result with the sixth clonal isolate and the six-times-cloned pool in Fig. 1 is that in Fig. 1 the independent passage series were started with a high-titer B virion pool prepared by one low-MOI passage of the sixth-clone virus from the plaque. It must be concluded that T particles were generated in small numbers during preparation of this pool and were amplified during each further undiluted passage.

Next, we considered whether a *ts* mutation in the B virion might alter these results, that is, whether the *ts* mutation dictates the generation of only one kind of T particle. Figure 2 shows that this is not so, since a clonal isolate picked directly from a plaque of the *ts* 31 mutant generated different T particle sizes in different passage series. None of these generated the one-fifth-genome-sized short T particle characteristic of the original *ts* 31 clonal isolate (22) (compare with *ts* 31 of Fig. 1).

Finally, Fig. 2 shows that all the different T particles present in the various independent passage series of the wild-type clonal isolate are unable to compete equally with each other. The tube labeled MIX is a mixture of all the T particles and B virions present in wild-type tubes 1 to 6. Note that only two of the many different T particles present were replicated in visible amounts. This suggests that some T particles generated early in passage series are never visualized due to inability to compete with others that were generated at about the same time.

Selective effect of host cells type upon production of T particles during serial passage of B virions. It is well established that different host cell lines show different degrees of autointerference due to DI particles (3, 10, 13, 19, 21). We tested the selective effect of two passages in different cell lines on the T particles produced during a third passage in BHK-21 cells. The starting pool of B virions employed was the same high-titer, sixth-cloned, first-passage pool shown (in Fig. 1) to consistently produce (and hence to contain) small numbers of two moderate-sized T particles on third passage in BHK-21 cells. Figure 3 shows that this pool did so again when passaged three

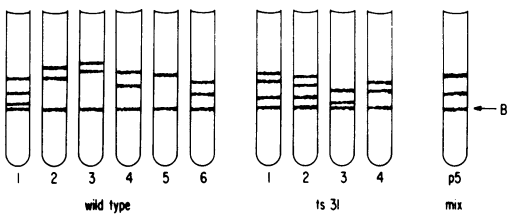

FIG. 2. *Generation of nonuniform sizes of T particles after independent serial undiluted passage series of B virions picked directly from isolated plaques of wild-type VSV or the ts31 mutant. Conditions for this experiment were identical to those for Fig. 1, except that here the starting B virions were those removed directly from isolated plaques with a Pasteur pipette and suspended in 1 ml of MEM. These were used to prepare six different wild-type (or four different ts31) clonal pools, each of which served as the starting inoculum in independent passage series of three more undiluted passages before analysis of the final yield as for Fig. 1. The tube labeled MIX shows the results of a competition experiment in which all of the different T particles present in wild-type tubes 1 through 6 were mixed together (1 ml of medium from each of the six final cultures was mixed), diluted 1/50, and used (1-ml amount) to infect 2 × 10^7 BHK-21 cells along with added B virions at a MOI of 100. The analysis of the yield after 20 h at 37 C is shown—only 2 of the T particles competed successfully enough to produce a visible band.*

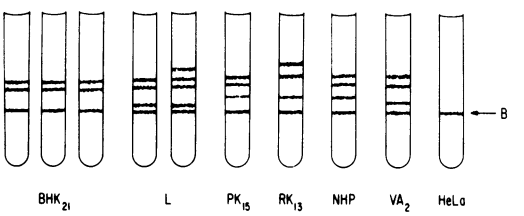

FIG. 3. *Production of nonuniform sizes of T particles from the same clonal B virion pool after serial passage in different cell lines. Conditions for this experiment were identical to those for Fig. 1, and the same frozen clonal B virion first-passage pool was used as inoculum for the first undiluted passage in the various cell lines. The first two serial undiluted passages were carried out in the indicated cell lines, and the third and final passage was in BHK-21 cells. The yield of the third passage in BHK-21 cells (displayed above) is the one that was purified and analyzed on sucrose gradients. BHK-21 cells were done in triplicate series of passages, and L cells were done in duplicate series. HeLa cells showed the same results after 2, 10, or 27 serial undiluted passages (i.e., no T particles visible after one further passage on BHK-21 cells). However, two serial amplification tests on BHK-21 cells showed that a very small number of T particles were present after 10 serial undiluted passages in these HeLa cells.*

times serially in BHK-21 cells, but whenever the first undiluted passages were carried out in a different cell line than BHK-21, followed by a final passage in BHK-21 cells, quite different arrays of T particles appeared. Note that L cells (mouse), PK15 (pig), RK13 (rabbit), and NHP (diploid human fibroblast) and VA_2 (SV40-transformed human fibroblast) generally amplified the same two T particle sizes seen in BHK-21 cells, but other sizes of T particles were also seen. Presumably these other T particles had little competitive ability during consecutive passages in BHK-21 cells but competed well and were amplified in serial passage in other cells (but different additional T particle types appeared in each case). It is likely that all these arose during preparation of the clonal stock B virion pool in BHK-21 cells. Obviously, the first to arise would have some advantage over the later-appearing T particles (the two T particles seen after serial passage entirely in BHK are also seen in most cases after passage in other cell lines), but some late-arising T particles and/or those with poor competitive value in BHK-21 cells obviously competed well during amplification in other cell lines. It should be noted that all of these were visualized after final passage on BHK-21 cells so all have some competitive ability in this cell.

Finally, we obtained the surprising result that no T particles were produced in serial passage in HeLa cells (not even after 27 consecutive passages in our HeLa cells followed by a final amplification passage in BHK-21 cells). This result and the above results point out once again the powerful effect of host cell factors on T particle replication.

Failure of HeLa cells to replicate added T particles in significant amounts despite strong T particle interference. Next we compared the ability of BHK-21 cells and HeLa cells to replicate added wild-type one-third-genome-sized T particles that had been purified from a BHK-21 cell yield. Table 1 shows that whereas our HeLa cell line produces comparable yields of B virions in the absence of T particles, they cannot replicate significant numbers of T particles (approximately 10,000-fold less than are produced by BHK-21 cells). Surprisingly, T particles interfered strongly with B virion replication in HeLa cells even though they replicated far less than the input T particle inoculum. This ability of T particles to interfere without replicating significantly should make these HeLa cells useful in studies of the biochemical basis of VSV DI interference.

Production of T particles during a single passage of rabies Flury strain on BHK-21 cells. Crick and Brown (7) have shown that serial passages of the HEP Flury strain of rabies virus produces T particles and we have confirmed this (12a). In view of the results obtained above with HeLa cells, we determined the number of undiluted passages necessary for cloned B virions to produce T particles. In contrast to VSV, B virions of rabies virus produced an array of different-sized T particles on the first passage in BHK-21 cells. Only B virions were visible 4 days after infection, but numerous T particles had appeared by 8 days postinfection (Fig. 4). This suggests that B virions are able to generate T particles in a single passage because they are poorly cytocidal (27, 17) and do not quickly kill infected cells as do VSV B virions. In fact, when we continue to cultivate BHK-21 cells infected at high MOI with rabies B virions, we consistently obtain carrier cultures (such as those described by Wiktor and Clark [32]) of persistently infected cells. These continuously produce both B virions and T particles (J. Holland and L. Villarreal, manuscript in preparation). This differs from VSV carrier states only in the absolute requirement of VSV for the presence of T

TABLE 1. *Inability of HeLa cells to replicate added VSV Indiana T particles as compared to BHK-21 cells*[a]

Input of virions/cell (PFU)	Input of T particles/cell (physical particles) (BHK-21 cells)	Yield of B virion/cell (PFU)	Yield of T particles/cell (physical particles)
BHK-21 cells			
200	0	19,000	0
200	1,000	80	5×10^4
HeLa cells			
200	0	11,000	0
200	1,000	150	$<10^6$

[a] Monolayers of 2×10^7 HeLa or BHK-21 cells were infected at indicated multiplicities followed by thorough rinsing to remove unabsorbed inoculum. B virions were measured as PFU on BHK-21 cells. T particles were measured as the number of physical particles based upon milligrams of protein in purified particles. A one-third-genome-sized wild-type T particle was employed here.

[b] No visible band of T particles could be purified from HeLa cell yields, but an amplification test of the supernatant medium at 24 h (on BHK-21 cells) showed the presence of from 2×10^1 to 8×10^1 biologically active T particles per cell. Over 90% interference with B virions yields was observed consistently in these HeLa cells.

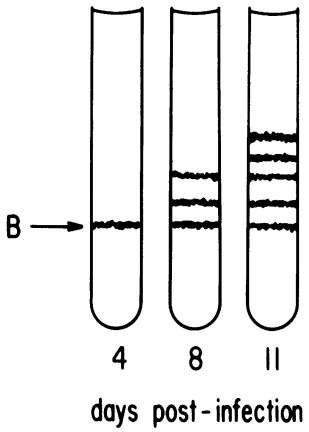

FIG. 4. *Production of T particles late during a single undiluted passage of a clonal B virion pool of rabies virus. Five monolayers of 2 × 10⁷ BHK-21 cells each were infected at a MOI of 30 on day 0 with a HEP Flury rabies clonal pool prepared by low-MOI infection of BHK-21 cells with B virions picked directly from a plaque in this laboratory (the plaque was generated by a three-times-cloned B virion pool supplied by H. Koprowski of the Wistar Institute). After adsorption of virus, the cells were incubated at 37 C for the indicated times, and the virus in the medium was purified and analyzed as for Fig. 1. At day 2 and every 48 h thereafter, the old medium was removed and replaced with fresh medium. From days 0 to 2, 4 to 6, and 8 to 11, MEM with 0.1% bovine serum albumin (27) was the medium employed, and from days 2 to 4 and 6 to 8, MEM with 5% calf serum was employed. This alternating regimen allows long-term maintenance of a single monolayer with some growth and allows maximal virus yields.*

particles at the onset of infection to establish a carrier state. We do observe massive cytopathic effects in BHK-21 cells at 7 to 15 days after rabies B virion infection, but surviving cells eventually reestablish nearly normal growth rates while producing T particles and B virions.

VSV T particle RNA is not able to replicate new T particles or cause interference in B virion-infected cells. Sreevalson (28) reported that RNA from VSV T particles caused interference. We were unable to repeat this observation. DEAE-dextran-facilitated uptake of T particle RNA caused no detectable interference (Table 2). Also, we employed very sensitive amplification testing on BHK-21 cells to determine whether B virion-infected cells can replicate any new T particles starting with added T particle RNA. Table 2 shows that they cannot. Even if only one in a million or less of the added T particle RNA molecules had replicated this would have been detected. It must be concluded that naked T particle RNA is biologically inert even though B virions presumably could provide all necessary replicating and encapsidating proteins. Likewise, Table 2 shows that RNA and DNA from cells replicating VSV and VSV T particles are unable to generate new T particles. All of these experiments were carried out under conditions where mengovirus RNA is maximally infectious.

TABLE 2. *Biological inactivity of T particle RNA, and of RNA or DNA from T particle-infected cells as detected by interference assays or amplification tests*[a]

Type of nucleic acid	Amt (μg) of nucleic acid exposed to 2 × 10⁷ BHK-21 cells	% Interference with B virion replication	Yield of T particles/cell (physical particles)
T particle RNA	162	<20[b]	<0.5[c]
Total RNA from cells infected with B virions and T particles	300	<20[b]	<0.5[c]
Total DNA from cells infected with B virions and T particles	270	<20[b]	<0.5[c]

[a] T particle RNA was obtained by phenol-chloroform extraction of purified T particles followed by repeated ether extraction to remove phenol and then by N_2 bubbling to remove ether. RNA was obtained from BHK-21 cells infected with approximately 100 PFU of B virions and 10 T particles per cell) at 20 h postinfection by phenol-chloroform extraction of cells suspended in 0.15 M NaCl followed by repeated ethanol precipitation of the aqueous phase. DNA was obtained from the interphase by addition of 1 mg of proteinase K per ml in 0.15 M NaCl, 1% Sarkosyl, and reextraction with phenol after digestion for 5 min at 60 C. DNA was repeatedly spooled from ethanol precipitates and redissolved. Nearly all T particle RNA extracted by phenol-chloroform remained intact (1.4 × 10⁶ daltons). All nucleic acids were exposed to 2 × 10⁷ BHK-21 cells at the indicated concentrations in 0.15 M NaCl immediately after the monolayers had been rinsed with 5 ml of 300 μg of DEAE-dextran per ml in Hanks balanced salt solution at pH 7.4. After 12 to 15 min of adsorption, the monolayers were rinsed and infected with B virions at a MOI of 10 and incubated for 24 h at 37 C in MEM.

[b] <20% Interference means no detectable reduction below the yield of 7 × 10⁸ PFU/ml in control cells mock-exposed to NaCl and dextran without nucleic acid.

[c] The yield from each experiment was analyzed directly, and no visible band of T particles was seen. In addition, 2 ml of the yield from each experiment was adsorbed to each of three bottles in an amplification test capable of 20,000-fold amplification of any T particles produced. Since 10¹¹ T particles constitute a faintly visible band (Holland and Villarreal, in press) and none was visible, the 2 × 10⁷ BHK-21 cells infected with B virions plus nucleic acid replicated less than 0.5 particles per cell (and therefore less than 1 cell in 10⁴ replicated T particles).

RNP cores from VSV T particles are able to replicate and to interfere in cells infected with B virions. In contrast to T particle RNA, T particle RNP cores are biologically active. Table 3 shows that, in the presence of DEAE-dextran to facilitate uptake, RNP cores are able

to replicate new T particles and to interfere in B virion-infected cells. The retention of biological activity by T particle RNP is not unexpected since Brown et al. (2) showed that B virion RNP is infectious. Table 3 also shows that substantial numbers of biologically active T particle RNP are present in the cytoplasm of BHK-21 cells 20 h after infection with a mixture of B virions and T particles. However, it can be seen that there is more than 1,000-fold less biologically active RNP in the cytoplasm of HeLa cells similarly infected for 20 h. It can be concluded that the failure of HeLa cells to replicate T particles (Table 1) is not merely due to a failure of encapsidation of active T particle RNP.

Ultrasonic fragmentation of B virion RNP or T particle RNP does not generate new short T particles. Since nothing is known about mechanisms of T particle generation, the above finding of replicating capacity in RNP coupled with a sensitive amplification test allows us to determine whether short T particles can be generated by shearing of B virion RNP or of long T particle RNP. Table 4 shows that although sonic fragmentation of B virion or T particle RNP gradually destroys biological activity of either, no new short T particles are seen after a 20,000-fold amplification on BHK-21 cells or even a 400 million-fold amplification in the case of fragmented B virion RNP. This suggests that random breakage is not the mechanism of T particle generation.

Failure of UV radiation or chemical mutagens and other inhibitors to influence T particle generation. We next attempted to influence the patterms of T particle replication by treatment of a B virion pool with mutagens. We mutagenized first-passage clonal pools of B virions (which contain only seed quantities of T particles) to determine whether mutagens and nucleic acid inhibitors can alter the predetermined pattern of T particles that would become visible upon serial passage. Table 5 shows that they do not. Neither direct mutagenization of the virus pool with UV light or nitrous acid (at an average level of slightly more than 1 hit per virion) nor the inclusion of 5-fluorouracil, cordycepin, 8-azaguanine, or rifampin in the medium during the initial high-multiplicity passage altered the size or ratio of the T particles appearing after the third serial passage. Obviously, T particle size is a stable characteristic in first-passage pools from clones. It was not possible to test the effect of mutagens on generation of T particles de novo by B virions picked directly from plaques since this is a

TABLE 3. *Biological activity of T particle RNP from T particle virions and from cytoplasm of infected cells*[a]

Source of RNP	Amt of RNP	% Interference with B virion replication	Presence of T particles in the yield as assayed by:	
			Direct purification from medium	One-cycle amplification test[b] (highest positive dilution)
Purified B virions	From 10^{12} PFU	<20	None	None
Purified T particles	From 10^{11} physical particles	99	+	+ (10^3)
Infected cell cytoplasm of BHK-21 cells	From 10^7 cells infected 24 h with B and T particles at MOI = 10 each	97	Not done	+ (5×10^3)
Infected cell cytoplasm of HeLa cells	From 10^7 cells infected 24 h with B and T particles at MOI = 10 each	<20	Not done	+ (Undiluted)

[a] Ribonucleoprotein was prepared from 10^{12} PFU of purified B virions or 10^{12} purified T particles by treatment with 0.1% Nonidet P-40 and sedimentation of RNP in a 5 to 40% sucrose gradient. The recovered bands were exposed to 2×10^7 BHK-21 cells (after appropriate dilutions of the T particle band) in 1 ml of 0.15 M NaCl immediately after rinsing the monolayers with 5 ml of 300 μg of DEAE-dextran per ml in Hanks balanced salt solution, pH 7.4. After 12 to 15 min of adsorption, the monolayers were rinsed with balanced salt solution and infected with B virions at a MOI of 10 and incubated for 24 h at 37 C in MEM. The yield of T particles was measured directly after purification (except where an amplification test was carried out in the B virion RNP experiment as for Table 2). BHK-21 cell or HeLa cell cytoplasm RNP were released by brief (2- to 5-s) ultrasound disruption of 24 h-infected cells suspended in 0.15 M NaCl. Dilutions of cytoplasm were tested for biologically active RNP levels with dextran-facilitated uptake as above.

[b] Amplification test results were recorded as positive if a visible band of T particles was observed (over 10^{11} particles). Numbers in parentheses are the highest dilutions of 2 ml of medium or 1 ml of cytoplasm that produced a visible band in this test.

TABLE 4. *Failure of sonic fragmentation of long T particle RNP or B virion RNP to generate new T particles detectable by replication of fragmented RNP in B virion-infected cells*[a]

Source of RNP	Time of ultrasonic shearing (s)	% Survival of replicative ability of T particle RNP after shearing	Approx no. of hits in T particle RNP	Size of T particles seen after amplification tests
Purified long T particles	60	ca. 50	<1	Long T
Purified long T particles	140	ca. 0.1	ca. 2	Long T
Purified long T particles	300	ca. 0.05	ca. 3	Long T
Purified B virions	140	—	—	None observed

[a] RNP particles of B and T virions were prepared as for Table 3. They were subjected to ultrasonic shearing from the times indicated. A setting of 2 was employed with the Branson model LS 75 sonifier with a thin probe. Surviving replicative ability of T particle RNP was determined by measuring T particles produced by RNP in a one-cycle amplification test (as for Table 3). Sonicated B virion RNP were amplified 10^8-fold by two consecutive amplification test cycles with added B virions. The sedimentation rates of T particles produced by replication of RNP were determined on sucrose gradients as for Fig. 1, and the long T particles replicating from sonicated RNP showed the same sedimentation velocity as the original long T particle. The reduced yields of this long T particle after shearing of RNP indicates that only surviving unsheared RNP were capable of replication with support B virions. The shearing capacity of the ultrasonic treatment employed here was verified by showing that 140-s shearing of ^{3}H-labeled B virion RNP converted most of the 40*S* virion RNA to heterogeneous subgenomic RNA sizes of less than 10^6 daltons.

random and unpredictable process even without mutagenesis (see Fig. 2).

Comparative target sizes of B virions and T particles. Because B virions have larger RNA than T particles, T particles should exhibit a smaller target size to UV light or other mutagens. Furthermore, they apparently do not transcribe RNA in vivo or in vitro (8, 14, 20, 24, 23) and might be resistant to mutations that would be lethal for B virion-coding function. Figures 5A and B demonstrate that T particles are considerably more resistant than B virions. A dosage of UV light that caused an average of 10 hits per B virion caused an average of slightly more than 1 hit per T particle with the one-third-sized T particle population employed. This is a greater resistance than can be explained by T particle RNA size, and it does suggest that significant portions of T particle RNA are indifferent to mutagenesis. Still, the target size represents more than a trivially small portion of the RNA.

Resistance of VSV B virions and T particles to photosensitization by intercalating dye. Since both B virions and T particles contain single-stranded RNA within helical nucleoproteins, we determined whether the RNA of either contained any degree of base pairing that would allow dye intercalation during replication and assembly. Virions containing RNA with base-paired secondary structure will intercalate proflavine during growth in the dark, and the virions are exquisitely sensitive to visible light photosensitization (18, 25). Table 6 shows that neither B virions nor T particles of VSV are rendered photosensitive by growth in the presence of 2 μg of proflavine per ml, although mengovirus grown under the same conditions is light inactivated by over 99.99%. We conclude that T particles and B virions both contain RNA in a nucleoprotein structure that prevents significant levels of RNA base pairing.

DISCUSSION

The variable T particle generation with direct clonal isolate B virions, together with the constant T particle generations by first-passage clonal pools of B virions, explains a previously very puzzling phenomenon. The type of T

TABLE 5. *Failure of mutagens and nucleic acid inhibitors to alter the T particles produced by a clonal pool of B virions*[a]

Mutagen employed	B virion survival (% of control yield of B virions at first passage)	Size and ratio of T particles produced during the third undiluted passage after mutagenization
Control, no mutagen	100	None
UV[b]	30	2 T[c]
HNO_2[b]	25	2 T[c]
5-Fluorouracil. 100 μg/ml	10	2 T[c]
Cordycepin, 100 μg/ml	12	2 T[c]
8-Azaguanine, 12 μg/ml	34	2 T[c]
Rifampin, 20 μg/ml	20	2 T[c]

[a] 5-Fluorouracil, cordycepin, 8-azaguanine, and rifampin were added to the medium of 2×10^7 cells infected at a MOI of 200 with the same clonal pool of wild-type B virions as for Fig. 1. After this first undiluted passage in the presence of mutagens, the B virion yields were measured and two further serial undiluted passages were carried out, then the third undiluted passage yields were purified as for Fig. 1 and the sizes and ratios of T particles were determined in sucrose gradients.

[b] For UV and nitrous acid mutagenization, the B virions of the clonal pool were UV irradiated to 30% survival (see Fig. 5A) or deaminated with nitrous acid to 25% survival (see Fig. 5B) before infecting cells in the first serial undiluted passage.

[c] Two T particles of moderate size (identical to those generated by wild type in Fig. 1) were produced.

TABLE 6. *Failure of proflavine to photosensitize B virions or T particles of VSV*[a]

Virions obtained from proflavine-treated cells	Proflavine in medium during virus replication (μg/ml)	PFU/ml in the dark	PFU/ml after light exposure	% Inactivation
Mengovirus (control)	2	1.7×10^9	8×10^6	>99.9
VSV B virions	2	6.1×10^9	5.8×10^9	<10
VSV T particles[b]	2			<20

[a] Viruses were grown in BHK-21 cells in MEM containing 2 μg of proflavine sulfate per ml for 20 h in total darkness to allow proflavine to intercalate into any RNA-containing secondary structure (18, 25). Virion and T particle yields were freed of unbound proflavine by passing the medium through columns of Bio-Gel A5 M (6 × 5 cm) in the dark and recovering virus in the void volume. One sample of recovered virus was assayed in the dark, and another was assayed after 15 min of irradiation at 0 C with visible light near a fluorescent lamp.

[b] T particle viability after light irradiation was determined by amplification tests (as described for Table 4) after dilution. No difference in replicative ability was seen between light-exposed T particles and those kept in the dark.

particle generated by cloned wild-type and mutant B virion pools was constant and appeared to be a genetic characteristic of B virions (22). Yet B virion clones reisolated from clonal stocks generated different types of T particles, suggesting enormous genetic instability of this apparent genetic character (19). The present results show that T particle generation is a random process occurring during first passage of a clonal isolate and resulting in "seeding" of particles in small numbers in first-passage pools. We have shown elsewhere that VSV Indiana B virions will amplify limiting numbers of T particles approximately 10^4-fold during each passage on BHK-21 cells (12a). If only a few hundred (or less) T particles are present in a first-passage pool, it is clear that two additional high-multiplicity passages will not provide the 10^{11} to 10^{12} T particles necessary to provide a visible band on a sucrose gradient, especially since amplification factors decrease as the T particle multiplicity exceeds 1 (12a).

In view of the above findings, it is clear that no B virion pool can be claimed to be free of T particles simply because it does not generate interference or visible T particle bands on the next two passages. This is very important in studies of the biological effects of T particles. For example, when we observed that rabies B virion clonal pools generated persistent noncytocidal carrier infections in BHK-21 cells (after extensive cytopathology), it appeared that T particles might not be required to establish rabies carriers as they are for VSV carriers (12). However, it is now clear that rabies clonal B virion pools are always contaminated with T particles. It takes a week to produce a small, turbid plaque of HEP Flury rabies on BHK-21 cells (27) and it is likely that T particles arise during this long-term plaque growth. It is likely that with many slower-growing viruses, isolated plaques will already contain T particles. In any case, the best method presently available for determining whether a B virion pool is free of T particles is to determine whether independent serial passage series from that pool always generate the same or different kinds of T particles.

The nature of the event(s) generating random sizes of T particles during first passage is a matter of speculation at present. However, trivial explanations such as virion RNA chain scisson or premature termination during synthesis seem rather unlikely in view of our inability to modify T particle sizes of RNP by sonic shearing (or with mutagenic agents). It may be that both the 3′ and the 5′ ends of the B virion and T particle RNA must be conserved for initiation of replication of nascent positive and negative strands, respectively. This would favor a recombinational mechanism of T particle generation in VSV as appears to be required for poliovirus DI generation (4). The UV inactivation kinetics for T particles (Fig. 5A) indicate that the RNA target size is not extremely small. The fact that T particle size distributions do not necessarily shift toward smaller size populations during serial passages in vitro (even in the presence of the chain-terminating drug cordycepin or mutagens) or during serial passages in newborn mouse brain (12a) argues against premature nascent chain termination as the mechanism of generation.

The failure of T particle RNA to replicate in cells where B virions are providing all necessary proteins is rather unexpected. Perhaps T particle RNA can be assembled properly within a nucleocapsid only as a growing RNA chain being extruded from template RNP. It is not clear why Sreevalsan (28) observed interference with T particle RNA, but the two-cycle amplification assay employed in the present study is thousands of times more sensitive than interference assays, and we have tested RNAs from several different kinds of T particles (in addition to the one reported here) with no evidence for biological activity.

The results obtained here suggest caution in comparison of results from different laboratories studying DI particles of mutant or of

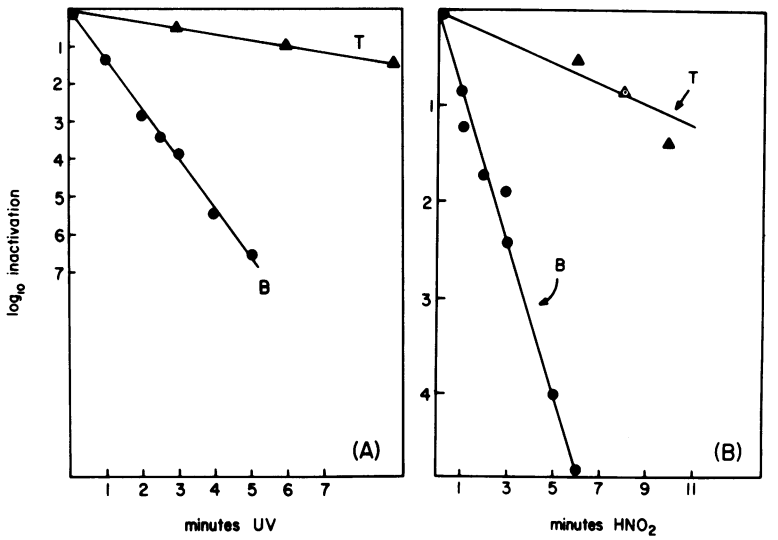

FIG. 5. *Comparative kinetics of inactivation of VSV B virions and T particles by UV light and by nitrous acid mutagenesis. (A) Purified B virions or T particles in 1 ml of TEN buffer were exposed to UV light for indicated times at a distance of 56 cm from a UVS-54 mineralite lamp. Surviving PFU were determined by plating 0.1 ml of various dilutions on 32-oz (0.95-liter) bottle monolayers of 2 × 10⁸ BHK-21 cells. (A large number of cells were required to reduce toxicity of concentrated dead virions at the higher UV dosage levels.) Surviving T particles were determined by quantitative amplification tests on appropriate dilutions of the irradiated T particles as compared to unirradiated controls. (B) Nitrous acid inactivation of B virions and T particles was carried out by adding 0.25 ml of a 1 M solution of $NaNO_2$ and 0.25 ml of a pH 4.5 1 M sodium acetate buffer to 0.5 ml of purified virus suspended in 0.15 M NaCl and incubating for the indicated times at 25 C. The fraction of surviving B virion PFU and T particle replicating units was determined after appropriate dilution as above for UV inactivation.*

wild-type VSV Indiana. It does appear that the most commonly arising T particle from wild-type B virion pools is one-third of genome size (23) and it is clear that different cell types may select for certain sizes of T particles. In addition, certain B virion mutants may also select for certain types of T particle (the data of Fig. 2 with *ts*31 show that this is not necessarily so); but it is also clear that T particle generation is somewhat random. Therefore, the wild-type T particles in different laboratories may well be different in size and in genome representation. Obviously, for different laboratories to ensure that they are generating and studying the same T particle they should employ the same first-passage (or higher-passage) B virion pool and utilize the same host cell line.

Finally, the high frequency and rather random nature of T particle generation in VSV make it highly probable that nearly all high-titer B virion pools contain at least some T particles. This probability, plus the results from the T particle competition experiment (Fig. 2) and the selection of different T particles in different cells (Fig. 3), suggest that T particle pools exhibiting a defined pattern of one or several T particle types visible on a sucrose gradient probably contain other T particle types in numbers too low to be visualized.

ACKNOWLEDGMENTS

We thank Estelle Ashcraft for her excellent technical assistance and H. Koprowski for providing the clonal rabies HEP Flury strain employed here.

This investigation was supported by Public Health Service grant CA 10802 from the National Cancer Institute.

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