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Suppression of Vesicular Stomatitis Virus Defective Intefering Particle Generation by a Function(s) Associated with Human Chromosome 16

C. YONG KANG,[1]* LAMONT G. WEIDE,[1] AND JAY A. TISCHFIELD[2]

Department of Microbiology, University of Texas Health Science Center, Dallas, Texas 75235,[1] and Department of Anatomy, Medical College of Georgia, Augusta, Georgia 30912[2]

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Human-mouse somatic cell hybrids were made between adenine phosphoribosyltransferase-deficient mouse L cells and a strain of human primary fibroblasts and selected in medium containing alanosine and adenine (J. A. Tischfield and F. H. Ruddle, Proc. Natl. Acad. Sci. U.S.A. **71**:45-49, 1974). These hybrids were tested for the generation of defective interfering (DI) particles of vesicular stomatitis virus to determine whether or not a host gene controls the induction of DI particles. None of the seven independently arising hybrid clones tested generated detectable DI particles during 13 successive undiluted passages. In addition, the parental human cells also failed to generate DI particles. In contrast, the parental mouse cells generated a detectable level of DI particles during continuous passage. Thus, failure to generate DI particles appears to act in a dominant fashion in these hybrids. Human chromosome 16 and adenine phosphoribosyltransferase were present, as a direct consequence of the selection system, in all of the hybrid clones that failed to generate DI particles. It was the only human chromosome observed in the cells of every hybrid clone. This was verified by both isozyme and karyotype analyses. After hybrids were back-selected (with 2,6-diaminopurine) for loss of human adenine phosphoribosyltransferase and chromosome 16, they gained the ability to generate DI particles. Replication of DI particles already present in virus stocks, however, was normal in all of the hybrid clones and the parental human cells. This suggests that the induction, but not the replication, of DI particles is affected by the human genome and that a factor on human chromosome 16 seems to selectively suppress the mouse cell's ability to generate DI particles in the hybrids. These results support the idea that the induction of DI particles is controlled in part by host cell function(s), as suggested previously (C. Y. Kang and R. Allen, J. Virol. **25**:202-206, 1978).

Many animal cells infected with RNA or DNA viruses have been shown to produce not only standard infectious virions but also defective virus particles which interfere with the replication of their parental infectious viruses. This phenomenon is known as homologous viral interference. The existence of defective particles in an animal virus was first described by Von Magnus in an influenza virus system (30). Since this discovery, information has accumulated on the generation of defective particles in many other virus systems. These defective particles are biologically active in interfering with the replication of their standard virus; hence, they were named defective interfering (DI) particles (14). It has become increasingly apparent that DI particles probably occur in most, if not all, animal viruses (12, 15). Huang and Baltimore (14) postulated a role for DI particles in host defense mechanisms involved in overcoming viral infections.

Koprowski and associates (3, 6, 7) have shown that C3H/He mice which are genetically resistant to infection with flaviviruses produce small amounts of the viruses after infection, both in vivo and in vitro. Brain explant cultures from resistant mice, infected in vitro with West Nile virus, yielded 100-fold less infectious virus than susceptible cultures. Spleen and peritoneal extracts and embryo fibroblast cultures prepared from the resistant mice all had diminished ability to support the replication of the flavivirus. Thus, the genetically determined resistance to flavivirus infections appears to involve factors controlling viral multiplication.

Darnell and Koprowski (3) conducted experiments to elucidate the mechanism whereby resistant cells are able to minimize production of

the virus. They demonstrated that infection with West Nile virus of cell cultures derived from inbred C3H/He mice genetically resistant to flavivirus infection produced a lower yield of the virus than did infection of cultures from the congeneic inbred C3H/He mice, which are genetically susceptible to flavivirus infection. They reported that the culture media obtained at the serial undiluted passages of the West Nile virus in the resistant cells interfered with the multiplication of the virus, whereas interference was not evident with comparable media from susceptible cultures. They also presented evidence that resistance was not due to the production of interferon. These results suggest that cell cultures derived from the inbred mouse strain which is genetically resistant to flavivirus infection generate DI particles regularly, but that cell cultures derived from the inbred mouse strain which is genetically susceptible to infection are incapable of DI particle generation.

Another role for DI particles may be evident from their association with the establishment and maintenance of persistent viral infections (31). Welsh et al. (31) have demonstrated that DI particles of lymphocytic choriomeningitis virus (LCMV) prevented central nervous system diseases produced by LCMV in rats. These authors further demonstrated that the expression of viral antigens on the surface of the LCMV-infected mouse cells was blocked by DI particles of LCMV. They postulated that DI-particle-mediated interference with viral protein synthesis may allow cells to escape immune surveillance during persistent infection. The role of vesicular stomatitis virus (VSV) DI particles in the establishment and maintenance of persistently infected cultures of BHK-21 cells has also been documented (8, 9, 11). These workers suggested that DI particles modulate the usually lethal cytopathic effects of VSV to allow indefinite survival of infected cells, which then multiply at normal or near normal rates.

Although the above-mentioned biological functions of DI particles have been documented, we know little about the generation of DI particles during the replication of the standard virus. Viral determinants have been suggested to be the only factors that influence the genesis of DI particles. For example, DI particles may be generated from virus as the result of premature termination during RNA replication, improper cleavage of the messenger RNA, the copying of the nascent RNA chain during the replication of the genomic RNA, or other mutational events during virus replication (12, 13, 15). However, Holland et al. (10) failed to generate DI particles by mechanical shearing of the ribonucleoprotein complex of VSV or by chemical and UV mutagenesis.

There is evidence which suggests that the clone of the virus may not be alone in determining the type of the DI particle produced, but that the host cell also plays an important role. Experiments by Choppin indicate that the amount of influenza virus produced is significantly dependent upon the particular host cell used (1). Madin-Darby bovine kidney (MDBK) cells produced a high yield of influenza virus in both high and low multiplicities of infection, whereas embryonated eggs produced a low yield of the virus with high multiplicities of infection. Choppin and Pons (2) demonstrated that MDBK cells produce influenza virions which contain at least five RNA species, whereas under the same conditions, HeLa cells produce a noninfectious virion deficient in the longest piece of the viral RNA. These results indicate that MDBK cells are either incapable of generating DI particles or that they may require many more serial passages to generate a detectable level of DI particles from the standard influenza virus. These results implicate host cell dependence in DI particle formation. Similar observations were made in the Sindbis virus system. BHK-21 and chicken embryo fibroblasts readily generate DI particles of Sindbis virus, whereas *Aedes albopictus* cell cultures fail to generate detectable DI particles (16). Kingsbury and Portner (20) demonstrated that different tissues within the same species respond differently in the generation of DI particles of Sendai virus. Whereas embryonated eggs readily generate DI particles, chicken embryo lung cells do not generate a detectable level of Sendai virus DI particles. Roman and Simon (25) have observed a similar phenomenon with Newcastle disease virus. Newcastle disease virus serially passaged in chicken embryo fibroblasts gives rise to DI particles, whereas the same virus passaged in embryonated eggs does not. These data suggest that not only do individual host species respond differently in the generation of DI particles, but also that particular stages in the differentiation of the same species may respond differently.

Although indirect evidence suggests that a host function(s) is in some way involved, we know very little about DI particle induction during undiluted serial passages of standard virus particles with high multiplicities of infection. We now have additional evidence implicating some host cell function(s). We found that the suppression of host cell functions by actinomycin D treatment before infection prevented the induction of DI particles of VSV (18).

To further substantiate host cell dependence

in the generation of DI particles, we determined whether or not independently derived human-mouse somatic cell hybrid clones behaved differently in the generation of DI particles. We reasoned that since independently derived human-mouse hybrid clones might contain different constellations of human chromosomes (28), individual clones may or may not contain a human gene(s) involved in DI particle induction. The experiment was believed to be feasible since mouse and human cells normally generate VSV DI particles with different sizes (19). Each hybrid clone was treated with 1 μg of actinomycin D per ml for 24 h, infected with VSV, and then overlaid with agar. The next day we picked the plaques and made three consecutive plaque isolations in cells pretreated with actinomycin D as described previously (18). The VSV plaques from the last plaque isolation were propagated in individual hybrid clones, which had also been pretreated with actinomycin D. At 8 h after infection, the viruses were harvested from each hybrid clone and used as the initial stock for high multiplicity of infection passages. The seven different hybrid clones generated no detectable DI particles during 13 consecutive high multiplicity of infection passages (Fig. 1b). This was an unexpected result since most cells in culture produce DI particles under such experimental conditions. Thus, we examined the parental human and mouse cell lines to determine whether they were capable of DI particle generation. To our surprise, the human parental cells did not generate DI particles, whereas the mouse parental cells did (Fig. 1a and 2). Our observation of no DI particle generation in the human cells is similar to an observation made by Holland and co-workers (10), who showed that a subline of HeLa cells is incapable of generating DI particles of VSV. These data suggest that there may be two separate functions in the host cell which control the generation of DI particles. One function may control induction, whereas a second function may be responsible for the suppression of induction of DI particles. (The terms suppression and suppressor are used

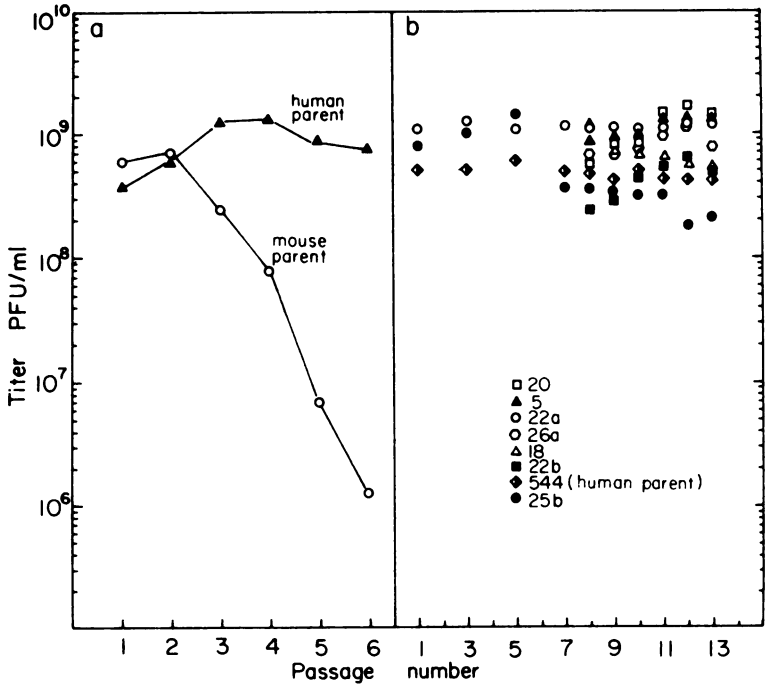

FIG. 1. *Yield of standard B virions during successive undiluted passages in seven different clones of human-mouse hybrid cells and in the cells from both parents. The initial stock virus was prepared by a consecutive plaque isolation method in cells pretreated with actinomycin D for 24 h. Confluent monolayer cultures of approximately 10^7 cells per 100-mm culture dish were infected with 1×10^9 PFU of the stock virus for the first passage. After 45 min of adsorption, 7 ml of prewarmed culture medium supplemented with 5% fetal calf serum was added to each culture dish and incubated at 37°C in a CO_2 incubator for 8 h. Cell-free virus fluids were harvested after 8 h of growth and centrifuged at 600 × g for 15 min. The subsequent passages were made by infecting 10^7 cells per dish with 0.5 ml of undiluted virus from the previous passage. The yield of infectious virus was measured by plaque assay of mouse L cells.*

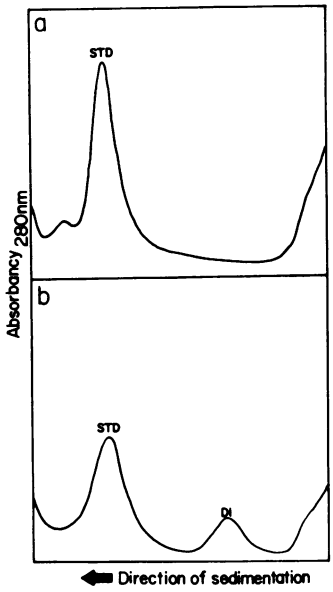

FIG. 2. *Analysis of total virus particles from the continuous undiluted passages of virus. Samples of lysate from two culture dishes of each passage illustrated in Fig. 1 were centrifuged at 600 × g for 15 min to remove cellular debris. The virus particles were pelleted from the supernatant by centrifugation at 81,000 × g for 75 min and in a Spinco SW27 rotor, suspended in 0.2 ml of phosphate-buffered saline, and layered on a linear 5 to 30% sucrose gradient made in phosphate-buffered saline. After centrifugation at 110,000 × g for 40 min in a Spinco SW41 rotor, the gradients were analyzed by a technique described previously (19). Positions of standard (STD) virion and DI particles are indicated. (a) Standard virion produced by the human parent and all human-mouse hybrid clones shown in Fig. 1b. (b) Standard virion and DI particles from the fifth passage of the parent mouse cells shown in Fig. 1a.*

without reference to any particular mechanism[s].) If the suppression acts as a dominant trait, that particular cell may not generate DI particles. Our data suggest that suppressor activity present in human cells can dominate the inducing activity of the mouse cell. Alternatively, perhaps there is only one host function (gene) involved in the control of DI particle induction, and the human cell line carries a mutant alloallele which acts in a dominant fashion to prevent DI particle induction. We do not know the mechanism(s) whereby this trait dominates.

Next, we determined whether there was any common human characteristic among the seven human-mouse hybrid clones. Since the parental mouse L cell was adenine phosphoribosyltransferase deficient ($APRT^-$) (29) and the human parent was $APRT^+$ diploid primary fibroblast strain 544 (fibroblasts obtained from the skin culture of a biopsy from the forearm of a normal female, courtesy of R. DeMars), we used the human fibroblasts in passages 20 to 35. The hybrids must have retained the human APRT gene, and all or part of human chromosome 16, to have survived in medium containing alanosine and adenine (28). The presence of human APRT was confirmed by gel electrophoresis (Fig. 3), and the presence of chromosome 16 was determined by karyotype analysis (28). The human-mouse hybrids were then tested for 28 different human enzymes which define 18 different linkage groups assigned to specific human chromosomes (22). Thus, the presence of a particular human chromosome in the hybrids could be directly inferred from the isozyme analyses. Only human chromosome 16, of all chromosomes tested, was present in all of the hybrid clones (Table 1). There were at least two hybrid clones among the seven that did not show one or more of the other human chromosomes tested for. This indicates a perfect concordance between human chromosome 16 and the failure to generate DI particles.

To confirm this association, we back-selected four human-mouse hybrid clones in medium containing 2,6-diaminopurine. This protocol selects hybrid subclones which have selectively lost human APRT activity and human chromosome 16 (28). The loss of APRT activity was confirmed by assay (26; data not shown) in five

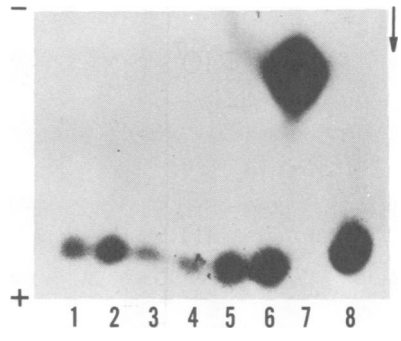

FIG. 3. *Autoradiograph of a starch gel showing APRT activity. Starch gels were electrophoresed with a citrate-phosphate buffer (pH 6.8) and incubated to show the migration of APRT activity after autoradiography (22, 27). Cell-free extracts of each of the hybrid clones and the mouse and human parental cell lines were placed in each well, as noted. Channels 1 to 6, hybrids P26a, P22b, P20, P18, and P5, respectively; channel 7, mouse L cell control ($APRT^+$, wild type); channel 8, human primary fibroblast strain 544. Only human APRT activity was detected in the hybrids.*

subclones. Isozyme analysis for 28 other human enzymes suggested that no other human chromosome was selectively lost in all of the back-selected hybrid subclones tested (data not shown). Thus, we created a series of subclones which, as a group, differed from the parental human-mouse hybrid clones only in the absence of human chromosome 16. We then performed similar experiments, attempting to generate DI particles of VSV in these five subclones. All five subclones produced DI particles, as shown by the reduction of the virus titer in the later passages (Fig. 4). Analysis of the cell-free lysate in sucrose gradients confirmed this (Fig. 5). Since all of these clones had selectively lost only human chromosome 16, this chromosome appears to contain the gene(s) responsible for the suppression of DI particle induction. It is also apparent that the sizes and number of DI particles generated in each of these hybrid clones differ significantly. It has not yet been ascertained which chromosome of mouse cells is responsible for the DI particle induction in each of these clones.

We also examined the primary human-mouse hybrid clones (which are incapable of generating DI particles) after infection with a virus stock already containing DI particles to determine whether these hybrid clones are capable of amplifying DI particles. We took sucrose-gradient-purified DI particles which were grown in BHK-21 cells and mixed them with the standard in-

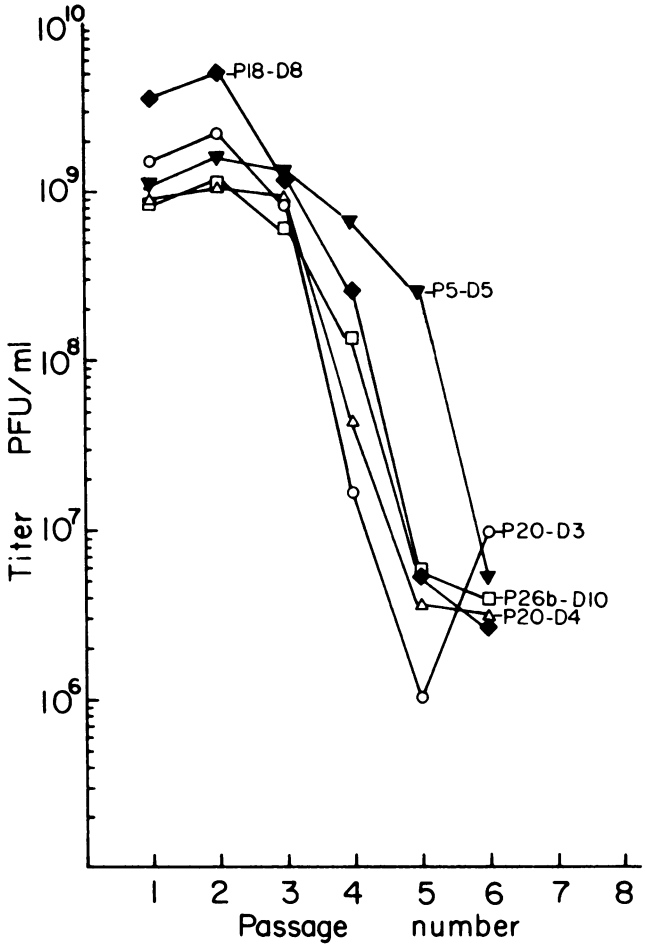

FIG. 4. *Yield of standard B virions during successive undiluted passages of VSV in five different clones of human-mouse hybrid cells lacking human APRT (marker for human chromosome 16). Virus samples from each passage were tested by plaque assay, as described in the legend to Fig. 1. P5-D5, P18-D8, P26b-D10, P20-D3, and P20-D4 were selected from clones 5, 18, 26a, 20, and 20, respectively. All five clones were selected for the loss of human APRT activity and the concomitant loss of human chromosome 16, by cloning in medium containing 75 μg of 2,6-diaminopurine per ml. Symbols are the same as on Fig. 2b.*

TABLE 1. *Genetic analysis of mouse-human hybrid clones*

Hybrid clones	Presence[a] of human chromosome no.:																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16[b]	17	18	19	20	21	22	X
22b	+	−			?	+		−		+	−	+		+	+	+		+	+	−	+		−
25b	+	+			?	+		−		+	+	+		+	+	+		+	−	−	+?		−
26b	+	+			−	+		−		+	−	+		+	+	+		+	+	+	+		−
5	−	−			−	−		−		−	−	−		−	−	+		−	+	−	−		−
18	−	−			−	−		−		−	−	−		−	−	+		−	−	−	−		−
22a	+	−			−	+		−		+	−	+		+	+	+		+	+	−?	+		−
20	+	+			−	+		−		+	−	+		+	−	+		+?	+	−?	+		−

[a] Symbols: +, chromosome inferred to be present; −, chromosome inferred to be absent; ?, uncertain scoring. The presence or absence of 28 different human enzymes was determined by starch gel electrophoresis (23).
[b] APRT marker.

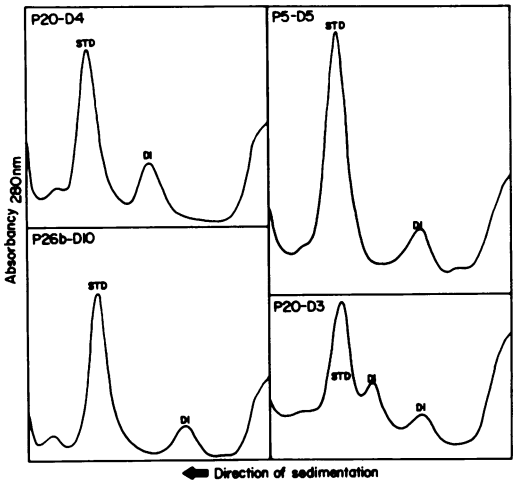

FIG. 5. *Analysis of total virus particles from the continuous undiluted passages of virus in human-mouse hybrid cells lacking human chromosome 16. Samples of lysate from two culture dishes of each passage illustrated in Fig. 4 were analyzed in the sucrose gradient, as described in the legend to Fig. 2. P18-D8 produced DI particles similar to those of P5-D5 and P26b-D10.*

fectious virus. We infected the human-mouse hybrid clones with this mixed virus. At 15 h after infection, the progeny virus was harvested and analyzed in the sucrose gradients as described previously (18). We found that the amplification of DI particles already present in an infecting virus stock was normal in all of the hybrid clones and in the parental human cells (data not shown). These hybrid clones behaved similarly to actinomycin D-pretreated cells (18). This suggests that only the induction, but not the replication, of the DI particles is affected by the human genome. The failure to generate DI particles in all of the primary hybrid clones was not due to low yield of virus since the parental mouse cells produced the same level of virus as the hybrids during the initial passages (Fig. 1a).

These results support our hypothesis that there are two different functions for DI particle induction, possibly alloalleles, operative in our hybrid cells: one function (mouse) is capable of inducing DI particles, whereas a second function (human) is responsible for the dominant suppression of inducing activity.

Speculation on the host function(s) responsible for the induction of DI particles and on the nature of the suppressor of the host function(s) is of current interest. Several investigators have proposed models to explain how DI particle genomes of RNA viruses could arise (13, 17, 21, 24). Models proposed by Huang (13), Perrault and Leavitt (24), and Leppert et al. (21) are concerned with the generation of panhandle-structured RNA, double-stranded RNA, and circular double-stranded RNAs. These investigators proposed that the virion RNA polymerase can copy the nascent RNA strand when the 3′ end of a nascent RNA molecule in some way turns around to form a hairpin. These models predict that the polymerase starts to copy toward the middle of the genomic RNA but at some point dissociates from the original template, turns around, and copies the nascent RNA chain in the direction of the 5′ end of the nascent chain. How a host function could modulate the viral RNA or the viral enzyme(s) or both is not clear.

Two other models explain the generation of simple linear single-stranded DI RNAs, which are abundant in nature (17). One of the models predicts that the host cell makes a loop of the template for RNA replication, enabling the RNA polymerase to read through without stopping at the loop junction. The looping of the RNA template and the copy choice of the RNA polymerase could generate the linear single-stranded RNA with internal deletions (4). The host function required for such events could be a molecule(s) necessary for loop formation of the viral RNA or ribonucleoprotein template.

Another possible mechanism for the genera-

tion of DI particles is host cell RNA processing. Many eucaryotic cell mRNA's arise from RNA processing after transcription, and mRNA processing events include a molecular splicing mechanism (5). Foreign RNAs, such as a virus RNA, might also be subject to the splicing mechanism(s). If a host cell enzyme(s) recognizes a specific site on the viral RNA and removes certain pieces of it by excision followed by ligation, a DI particle RNA could be generated. The enzymes involved in cutting the RNA and joining the ends may be the host functions responsible for the generation of the DI particle genome. This kind of RNA processing may frequently occur, but we can only detect it if such processed RNA can be amplified by a virus-specific RNA polymerase. An inhibitor of such RNA processing enzymes could prevent DI particle formation.

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