

EFFECTS OF DEFECTIVE INTERFERING VIRUSES ON
VIRUS REPLICATION AND PATHOGENESIS
in Vitro AND *in Vivo*

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I. INTRODUCTION

Molecular Nature and Origin of Defective Interfering (DI) Particles and Satellites—Recombination, Rearrangements, and Biological Activities

DI particles are subgenomic deletion mutants generated from infectious virus genomes, generally by replicase errors. DI particles and related satellite genomes of plant RNA viruses are generated by a

wide variety of animal, plant, and fungal viruses. For reviews see Blumberg and Kolakofsky (1983), Barrett and Dimmock (1986), Holland (1987, 1990), Huang (1988), Schlesinger (1988), Nuss (1988), Kaper and Collmer (1988), and Simon (1988). This chapter focuses on material not already covered in these earlier reviews. The ubiquity of DI viruses was first clearly recognized by Huang and Baltimore (1970). They proposed and defined the term "DI particle" to include defective viruses containing only some portion of the infectious virus genome, requiring homologous parental virus as helper for replication, containing virus structural proteins and antigens, and exhibiting the capacity to replicate preferentially at the expense of infectious helper virus in cells infected by both. In most cases DI particle interference results from competition for helper virus-encoded replication-encapsidation proteins. Because DI particles lack (greater or lesser) fractions of their parental virus genomes, they sometimes have mature virions which are detectably (or markedly) smaller in size than the parental infectious virions which generate them. In their original definition of DI particles, Huang and Baltimore did not include defective virus particles which are not subgenomic, for example, full-sized virus genomes which are defective because one or more mutations have closed essential open reading frames (ORFs). There is reason to suspect that such defective full-size virus genomes may sometimes exert interfering and other important biological effects, and this is discussed in Section II,B,3.

Satellite RNAs of plants are small RNAs which usually share little or no homology with their helper viruses, although some do exhibit some sequence homology with their helper viruses and thus resemble DI particles in at least a portion of their genomes. The origin of non-homologous satellite RNAs is unclear at present, although those with self-cleavage ribozyme activity may have evolved from primitive RNA precursors. [See Section III and reviews by Kaper and Collmer (1988), Simon (1988), and Bruening *et al.* (1988) for discussions of satellite RNAs.] All have in common the capacity for encapsidation within protein encoded by their specific helper viruses.

The molecular origin of most DI particles of RNA animal viruses is rather well established to be a consequence of polymerase errors, as originally suggested by Huang (1977) and Leppert *et al.* (1977). They suggested that DI genomes arise by virus genome rearrangements or recombinations as a result of viral replicases "leaping" or skipping from one virus RNA template to another or from one segment of a template to another. During this leaping (or skipping or sliding) the RNA replicase carries the incomplete nascent strand to a new template (or template segment), then uses this nascent strand as a primer for

resumption of chain elongation at the new template resumption site. Depending on the leaving sites and resumption sites, DI particles can be produced which are simple internal deletions of the virus genome, simple deletions with a new terminus, or complex or bizarre genomes with multiple rearrangements of virus segments. The leaping replicase mechanism for DI particle generation has been generalized by Lazzarini *et al.* (1981) and Perrault (1981). This type of "copy choice" recombination of virus RNA has been experimentally verified for polioviruses by Kirkegaard and Baltimore (1986) and for coronaviruses by Lai (1990) and colleagues, and was suggested for turnip crinkle virus satellite RNAs by Cascone *et al.* (1990).

It is obvious that promiscuous replicase leaps might insert cellular RNA into defective (or infectious) viruses, and, in fact, Monroe and Schlesinger (1983) demonstrated the incorporation of cellular transfer RNA into the 5' termini of a class of Sindbis virus DI particles (Schlesinger, 1988). Although available evidence favors replicase leaps as the major mechanism for the generation of DI particles, other mechanisms (e.g., aberrant splicing events) might rarely be involved. Because many DI particles of RNA viruses are replicative entities only, being incapable of transcription and translation of their genomes, only those genome segments necessary for efficient replication and encapsidation need be conserved. These DI particles can undergo extensive mutational (and recombinational) change, so repetitive mutational change or biased hypermutation (Cattaneo *et al.*, 1988a; Wong *et al.*, 1989) may frequently affect the structure of defective RNA genomes. Some hypermutation might be due to error-prone subsets of polymerase (O'Hara *et al.*, 1984a; Steinhauer and Holland, 1987) or to RNA unwinding enzymes which convert A residues to I (Bass and Weintraub, 1988; Lamb and Dreyfuss, 1989) or to any number of other novel phenomena of RNA genetics, such as RNA editing (Simpson, 1990) or the addition of nontemplated bases (Thomas *et al.*, 1988). The generation and recombination of plant DI and satellite RNAs are discussed in Section III.

DI particles of DNA viruses and retroviruses may also sometimes arise by replicative error, but probably most often are generated by the variety of genetic recombination mechanisms available for DNA (Kucherlapati and Smith, 1988; Berg and Howe, 1989) and retroviruses (Coffin, 1990).

1. Interference and Amplification

In general, DI particles are replicative entities. They replicate and amplify their genomes at the expense of the replication of specific helper viruses which encode replication and encapsidation proteins,

and which must compete with the DI genomes for these gene products. The genomes of the DI particles are usually rearranged to enhance their ability to replicate (and to compete for replication–encapsidation proteins). This results in selective replication and maturation of DI genomes and concomitant interference with the replication of infectious helper virus. For example, simian virus 40 DI genomes generally contain reiterated viral origins of DNA replication, and vesicular stomatitis virus (VSV) DI particles usually contain rearranged termini which favor replication and preclude transcription. Therefore, while helper virus genomes become devoted mainly to transcription, DI genomes engage only in replication. However, some classes of DI genomes are transcriptionally active, and these often interfere at a level other than replication. Sometimes DI particles interfere indirectly with virus, as when they induce interferon (IFN), alter immune responses, or restrict the cell surface expression of virus proteins. Finally, the presence or absence of encapsidation or packaging (sequences) determines the relative efficiency of maturation of DI genomes. All these parameters have been reviewed or discussed by Huang (1988), Schlesinger (1988), Holland (1987, 1990), Brockman (1977), Makino *et al.* (1990), Sekellick and Marcus (1980a, Barrett and Dimmock (1986), and Roux *et al.* (1984).

2. Influence of Host Cell Type

Host cell type is a major determinant of the biological effects of DI particles and one which is frequently overlooked in attempts to assess the influence of DI genomes on virus diseases *in vivo*. DI particles which interfere strongly in one host cell type may interfere weakly or not at all in other cell types. In some cases this is due to poor replication and amplification of the DI particles, but in other cell types DI particles may replicate well and amplify well, but exert only weak interference with helper virus replication. Variability of DI particle effects within different cells *in vivo* can be a major factor confounding interpretation of DI genome influences on disease processes. For reviews or discussion of host cell effects, see Choppin (1969), Huang (1988), Brinton *et al.* (1984), Cave *et al.* (1985), Kang *et al.* (1981), Holland (1987), Gillies and Stollar (1980, and Barrett and Dimmock (1986).

3. Assays for DI Particles

DI particles and DI genomes are generated by nearly all types of animal viruses, and by some plant and fungal viruses. They frequently contaminate animal virus pools *in vitro* and *in vivo*. Their presence is not always easily detected, depending on the virus assay system used,

the type of virus, the DI genome class, etc. There is no sensitive reliable assay system suitable for all virus and DI genome types, so a wide variety of DI genome assays have been developed. These have been reviewed by Holland (1987). They range from electron microscopy of particles or particle purification and visualization on sucrose velocity gradients, to assays for interference with virus-directed nucleic acid synthesis or virus particle synthesis, to assays for subgenomic-sized nucleic acids in particles, cells, or tissues, to various cell protection assays. Recent polymerase chain reaction (PCR) techniques for amplifying DNA and RNA segments should prove useful, but the choice of appropriate primers will be important (and difficult when the structure of the putative DI genomes present is not known).

4. Cell Protection and Persistence

Because DI particles often interfere strongly with virus replication, it was anticipated (Huang and Baltimore, 1970) that they might frequently exhibit a direct cell-sparing effect (in addition to indirect protective effects, such as induction of IFN or other cytokines, alteration of immune responses, reduction of early virus yields, or expression at the cell surface to allow immune abrogation of infection). The interfering effects of DI particles can be so profound as to reduce the virus yield to zero in many doubly infected cells (Sekellick and Marcus, 1980b). It has frequently been observed that DI particles can facilitate the establishment and maintenance of persistent infections of cells in culture infected by a wide variety of animal viruses. This has been reviewed by Holland *et al.* (1980), Holland (1987), Huang and Baltimore (1977), Schlessinger (1988), and Barrett and Dimmock (1986). It would not, therefore, be unexpected if DI genomes often played significant roles in reducing virus yield *in vivo*, thereby facilitating complete immune system elimination of virus in some cases, or triggering persistent infections in others. However, it is not a simple matter to test this possibility in any natural infection of animals or humans. This is discussed below, and by Huang and Baltimore (1977, Huang (1988), Barrett and Dimmock (1986), and Holland (1987). Of course, many other factors such as temperature-sensitive (*ts*) and other mutations can be involved in persistent infections (Youngner and Preble, 1980; Cattaneo *et al.*, 1988b; Wong *et al.*, 1989; Enami *et al.*, 1989). Even host cell mutations are sometimes involved in persistent infections (Ahmed *et al.*, 1981).

5. Cycling Phenomena and Effects on Virus Evolution

Palma and Huang (1974) reported a significant cyclical process in the interaction of helper virus and DI particles *in vitro* when they

interact continuously or repeatedly during repeated passages. They observed out-of-phase cyclic yields of each. Whenever DI particle yields were sufficient to infest most cells, the yield of infectious virus dropped sharply; then there was insufficient virus helper to support significant DI particle replication; then infectious virus yields increased again, only to decline once more as DI particles were amplified, etc. Evidence for cycling has been observed in many virus-DI interactions (Kawai *et al.*, 1975; Grabau and Holland, 1982; Roux and Holland, 1980), and it obviously could create uncertainty regarding DI particle (or virus) yields during any single sampling of a passage series, or an infected animal or human. Cave *et al.* (1984, 1985) have provided strong evidence that DI particles of VSV cause virus cycling *in vivo* in mice and that lower input ratios of DI particles are more protective than high ratios because of virus-DI cycling phenomena. This has profound implications for the stochastic nature of many virus infections, and for the severity and outcome of infections. The disease process might depend on probabilities of DI genome generation early in infection or on simultaneous infection by varying ratios of virus to DI particles (Huang, 1988). Bangham and Kirkwood (1990) have presented a simple mathematical model to explain virus-DI cycling effects on the replication and persistence of viruses.

Another type of virus-DI genome cycling can be superimposed on the basic type of cycling described above. The second type of cycling occurs during prolonged virus-DI genome interactions in persistent infections or serial passages, and it is due to the periodic unpredictable generation of DI particle-resistant (*Sdi-*) mutant viruses. The appearance of virus mutants resistant to homologous DI particles has been reported for a number of different RNA and DNA viruses, including rabies (Kawai and Matsumoto, 1977), VSV (Horodyski and Holland, 1980), lymphocytic meningitis virus (Jacobsen and Pfau, 1980), Sindbis virus (Weiss and Schlesinger, 1981), West Nile virus (Brinton and Fernandez, 1983), and DNA coliphage $\phi 1$ (Enea and Zinder, 1982). Giachetti and Holland, 1988) can allow the periodic escape of virus from interference effects of previously existing DI particles; then newly generated DI genomes interfere until virus mutates again, etc. (Horodyski *et al.*, 1983; DePolo *et al.*, 1987). This type of cycling can drive rapid virus evolution and lead to unpredictable periodic emergence of new virus and DI genomes (O'Hara *et al.*, 1984a,b). Obviously, if complex cycling phenomena of the types described above occur within multiple sites of virus infection in an animal or a human, then analysis of the role of defective genomes in the disease process can become enormously complicated.

6. *Involvement of Defective Virus Genomes in the Biology of Virus Disease Processes*

As discussed in Section III, DI and satellite RNAs of plants clearly can exert varying *in vivo* effects on plant disease symptoms, causing reduction of symptoms, no effects on symptoms, or mild to profound intensification of disease severity (see also Kaper and Collmer, 1988; Simon, 1988). The regions of these defective genomes involved in effects on plants are now being elucidated (see Section III). The situation is considerably more complicated in animals and humans because of their immune responses, neuroendocrine responses, more highly specialized tissue and organ systems, etc.

It is well established in animal models that DI genomes and other defective genomes can influence disease processes, but their possible roles in natural infections remain largely unexplored (Huang, 1988; Holland, 1987; Barrett and Dimmock, 1986). This is partly due to difficulties in obtaining suitable tissue specimens for defective virus isolation—especially from humans. It is also due to technical problems in proving or disproving a biological role for defective genomes even when they are observed in clinical material. Several examples discussed below illustrate the difficulty.

It required decades of careful research to achieve recognition that partially defective measles virus is involved in the persistent progressive fatal brain disease subacute sclerosing panencephalitis (Hall and Choppin, 1979; Cattaneo *et al.*, 1988a,b; Wong *et al.*, 1989; Enami *et al.*, 1989), even though a large proportion of central nervous system (CNS) neurons are producing measles virus antigens and virus genomes during disease progression. Multiple mutations accumulate within virus surface envelope proteins, particularly the matrix protein, yet virus genomes are somehow transmitted from cell to cell without significant production of mature infectious virus, and despite a vigorous CNS immune response. This type of defective virus is not a DI virus, because it replicates autonomously without helper virus. Whether helper-dependent DI genomes are present and replicating at the expense of these partially defective genomes is presently unknown.

A less definitive situation exists with hepatitis B virus (HBV). A number of investigators have observed inactivating mutations, particularly in the precore and core regions of virus genomes recovered from persistently infected humans (see Okamoto *et al.*, 1990; Miller *et al.*, 1990, and references therein). These mutations might prevent synthesis and secretion of hepatitis B antigen, thereby reducing the probability of immune destruction of chronically infected hepatocytes. Mil-

ler *et al.* (1990) showed that defective genomes are not necessary in a cloned recombinant inoculum to produce chronic hepadnavirus infections in woodchucks at a rate equal to, or higher than, that obtained with an inoculum containing defective genomes. This does not, of course, rule out regular participation of defective hepadnaviruses in disease processes or persistence, since defective genomes might be undergoing cyclical interactions with nondefective genomes in many liver foci of all infections, regardless of inoculum. In fact, Miller *et al.* (1990) showed that two of three recombinant DNA clones recovered from a woodchuck inoculated with a standard serum pool had mutational defects which rendered them incapable of independent replication. In a situation such as this, it is extremely difficult to prove or disprove a biological role for defective genomes even when they are observed repeatedly in clinical specimens. This also applies to hepatitis A virus (HAV) infections, in which defective subgenomic virus genomes have been observed by some investigators, but not by others (Nüesch *et al.*, 1988; Lemon *et al.*, 1985).

Despite extensive recent investigations of human immunodeficiency virus type 1 (HIV-1), it is still uncertain whether defective genomes might be involved in causing the acquired immunodeficiency syndrome (AIDS) disease process (or attenuating it). Defective genomes, of course, play a major role in most forms of animal retrovirus oncogenesis (Coffin, 1990). Huang *et al.* (1989) have shown that a strain of murine leukemia virus causing acquired immunodeficiency in mice can do so with a helper-free defective genome alone. Apparently, this defective retrovirus genome induces oligoclonal expansion of target cells to induce a paraneoplastic immunodeficiency. Myerhans *et al.* (1989) have shown that a considerable proportion of HIV-1 genomes may be defective due to inactivating mutations in the *tat*, *gag*, and *env* sequences. Balfe *et al.* (1990) reported a much lower level, but in neither case is it possible to affirm or negate a role of defective genomes in disease or disease attenuation. Finally, it should be recognized that the distinction between defective and nondefective genomes is not always clear. For example, it appears that there are subgenomic mRNA replicons present during the normal replication of full-sized coronavirus genomes (Sethna *et al.*, 1989, 1991). This might allow the subgenomic replicons to compete with standard genomes in a manner analogous to DI genomes, thereby potentiating the establishment of persistent coronavirus infections, as postulated by Sethna *et al.* (1989, 1991).

Even with powerful new analytical methods for virus genome analysis, it will take much time and effort to elucidate the involvement of defective genomes in virus diseases of humans and animals. Section II

reviews some recent studies of defective animal and human viruses, and Section III covers recent work with DI and satellite RNAs of plant viruses.

II. DI VIRUSES OF ANIMALS AND HUMANS

As emphasized above, defective genomes and DI particles have been studied widely in *in vitro* systems in which their ability to modulate the intensity as well as the course of viral infections has been extensively described for RNA and DNA viruses. Their ability to modulate infections in experimental animal models has also been documented and extensively reviewed (Barrett and Dimmock, 1986; Holland, 1987; Huang, 1988). However, despite this large body of *in vitro* and *in vivo* information from laboratory experiments, there is still no definitive confirmation (nor refutation) of their presence and involvement in natural diseases of humans and animals. This is due, partly, to lack of interest in the potent modulators of viral infections that defective particles represent, and, mainly, as emphasized in Section I, to the unpredictable complexity of DI multiplication in a noncontrolled system. Also, the inherent characteristic of defective particles which, by definition, do not efficiently replicate in low helper virus multiplicity of infection, has prevented easy isolation from natural infections. Direct analysis of naturally infected samples, on the other hand, has often suffered from the inability to distinguish components of defective viruses from those of nondefective viruses. With the availability of more sensitive techniques (e.g., PCR), this detection is bound to become more feasible, with the major drawback that PCR may allow definitive detection only of already described defective genomes. Indeed, such a sensitive technique may turn out to be misleading in cases in which the choice of the right primers cannot be backed up by previous information regarding defective genome structures (see Section II,A,9). In this section we try to give a nonexhaustive up-to-date view of certain aspects of defective virus research, focusing, when possible, on data dealing with the possible involvement of defective genomes in animal and human diseases.

A. RNA Viruses

1. DI RNA Generation

The mechanism of DI RNA generation implies the ability of the RNA replicase to jump from one template, or one portion of a template, to another, carrying the nascent RNA strand (see Section I). The ease

with which DI RNAs are then generated must therefore be inversely proportional to the processivity of the enzyme, that is, its ability to stick to its first template. Variant viruses have been described which are able to generate VSV DI RNA with a much higher efficiency (De-Polo and Holland, 1986). For such variants one would postulate that the replicase has a decreased "processivity," and/or a better ability to rebind to a new template. The protein responsible for controlling one of these two steps has apparently been identified for influenza virus (Odagiri and Tobita, 1990). A mismatch of the *NS* gene between two viral strains (*NS* of A/Aichi transferred to A/WSN by reassortment) has generated a strain (Wa-182) with the ability to produce detectable DI particles very rapidly (i.e., within a single high-multiplicity infection). This characteristic of Wa-182 was cotransferred to a third strain with the *NS* gene. Three point mutations in the Wa-182 *NS* gene were identified relative to wild type, resulting in two amino acid substitutions in the NS2 protein; NS2 is therefore likely to be a component of the replicase involved in the control of its processivity or its ability to continue chain elongation on a new template.

2. Interference at the Replicating Level

In vitro systems capable of VSV DI RNA replication have been worked out using infected cell extracts (Peluso and Moyer, 1983) or programmed reticulocyte lysates (Patton *et al.*, 1984; Wertz *et al.*, 1987). These systems, apart from giving interesting information on the viral RNA replication mechanism (Peluso and Moyer, 1988; Moyer, 1989), have allowed verification of a basic postulate of the interference mechanism (Perrault 1981; Holland, 1987). Competition between nondefective and defective genomes for availability of the *L/NS* complex to support efficient DI RNA replication has been demonstrated directly *in vitro* (Giachetti and Holland, 1989). This confirms previous *in vivo* evidence that DI particles interfere mainly at the level of replication (Huang and Manders, 1972; Perrault and Holland, 1972).

3. Packaging of DI Genomes

Competition at the level of replication is certainly the major factor explaining interference and successful amplification of DI genomes. Under multiple infectious cycles, however, packaging efficiency of DI genomes into virus particles may also intervene in their ability to outgrow nondefective or other defective genomes. It has long been recognized that if VSV DI RNAs are rapidly assembled into nucleocapsids they appear to be more slowly and less efficiently matured into virus particles (Palma and Huang, 1974; Khan and Lazzarini, 1977;

Moyer and Gatchell, 1979). This question has been reinvestigated (Von Laer *et al.*, 1988). Under low interference conditions [DI/ST standard virus = 0.125 in the inoculum] DI particles were, in fact, found to be delayed in their production relative to nondefective particles. Under higher interference conditions (DI/ST > 2), however, both ST and DI particles were found to be delayed in their production, with no particular handicap for DI nucleocapsids. This delay could then be correlated with retarded, but more prolonged, viral protein synthesis. By quantitative comparison of intracellular and extracellular Sendai virus copy-back DI nucleocapsids, restriction of DI nucleocapsid budding relative to ST nucleocapsid budding was observed, but only above a certain level of DI RNA replication (Mottet and Roux, 1990). These two examples show that DI nucleocapsid packaging can be modulated either temporally or quantitatively, depending on the conditions of interference. This may, in turn, influence the course of infections by modulation of the ratios of DI/ST in successive inocula. Restriction of DI nucleocapsid budding during excessive intracellular DI replication may prevent dying out of the infection due to lack of helper virus in the yields. Not all types of DI nucleocapsids are restricted in their packaging in the same way. Larger nucleocapsids appear to be less restricted than smaller ones (Mottet and Roux, 1990; Re and Kingsbury, 1988). This may be indicative of as yet unknown rules directing nucleocapsid packaging.

4. A Model to Account for DI Cell-Sparing Effects

Under conditions of high interference, decreased amounts of intracellular VSV M and G proteins have been observed together with prolonged synthesis of host proteins (Von Laer *et al.*, 1988). These conditions correspond to the cell-sparing effects following Sendai virus DI particle infections. Cell-sparing is observed together with a strong restriction of total virus particle budding (Tuffereau and Roux, 1988). Thus, it was proposed that negative-strand virus DI allows cell survival and the establishment of persistent infections by promoting reduced efficiency of viral particle assembly (Tuffereau and Roux, 1988). In this model it is the efficient and stable formation of virus prebudding structures (glycoproteins /M/ST nucleocapsids) at the plasma membrane which would otherwise lead to cell death. DI nucleocapsids would be unable to enter, or stabilize, these prebudding structures. Virus particle budding is then decreased (Tuffereau and Roux, 1988), and M, G, or HN (for Sendai virus) are decreased in amount (Tuffereau and Roux, 1988; Von Laer *et al.*, 1988) due to faster degradation (Tuffereau and Roux, 1988; Roux *et al.*, 1984). Any alterations (e.g., mutation or deletion) of proteins involved in the stable formation

of these prebudding structures would have similar cell-sparing effects on infected cells.

5. DI Particles and Interferon Induction

Cell-sparing effects produced by DI particles can be observed in the absence of any IFN production. IFN induction may, however, be another way by which DI particles can modulate the course of a viral infection. The ability of DI particle-enriched VSV inocula to promote IFN production has been recognized previously, but with ambiguity due to the ability of wild-type virus to induce IFN, on the one hand, and to inhibit host cell protein synthesis, on the other (reviewed by Holland, 1987). This complex question has recently been reexamined for VSV (Marcus and Gaccione, 1989). DI containing self-complementary (snap-back) RNA was shown to induce 20- to 30-fold higher amounts of IFN than plaque-derived VSV. This effect is heat resistant and thus likely due to the double-stranded nature of infecting DI RNA. In the absence of heat treatment, strong induction of IFN by DI was observed only at low multiplicity of infection (1–2 DI particles and 0.1–0.2 plaque-forming units). Higher multiplicities caused marked reduction in IFN yield. Since this reduction was not observed after heat treatment of DI stocks, the authors postulate that IFN reduction at higher multiplicity of infection results from the inhibitory effect of VSV on cell protein synthesis via small leader RNA synthesis (Grinnell and Wagner, 1984). What is noteworthy here, in the context of possible effects of DI on the course of viral infections in animals, is the minimal multiplicity of DI infection required to promote maximal effects on IFN induction. Thus, potential biological effects of DI in animal infections would not necessarily require massive doses.

6. DI Particles and Immune Response Modulation

Another effect of DI particles on animal infections is their puzzling ability to modulate the specific humoral immune response (McLain and Dimmock, 1989). Mice infected with lethal doses of influenza viruses produced high levels of antineuraminidase antibodies in the lungs. Coinfection with a lifesaving dose of DI viruses (but not with β -propiolactone-inactivated DIs) was accompanied by the appearance of not only antineuraminidase, but also hemagglutination-inhibiting antibodies. The latter show protective ability when passively transferred to naive mice before infection. These antibodies, entirely of the immunoglobulin G types, are nevertheless nonneutralizing antibodies and are found uniquely in the lungs (from which they disappear within 15 days). Their protective activity was postulated to be due to opsonization of virus or infected cells.

7. *DI Particles in Natural Infections*

A correlation was observed between high virulence of influenza virus epidemics in chickens, with the absence of DI particles produced by the viral strains implicated (Bean *et al.*, 1985; Webster *et al.*, 1986; Chambers and Webster, 1987). Involvement of DI particles in natural infections has also been supported by Northern blot analysis of RNAs extracted from specimens (feces and blood) of humans suffering from HAV infections. The presence of subgenomic viral RNA molecules was observed (Nüesch *et al.*, 1989). Interestingly, the identification of the deletion end points in these RNAs, by RNase protection experiments, is compatible with two of the three deletions identified in RNAs of HAV particles grown *in vitro* and shown to interfere with standard virus replication (Nüesch *et al.*, 1989). These HAV DI RNAs have been identified in all HAV-infected cell culture systems reported to date. The propensity of most of the *in vitro* infection systems (cited by Nüesch *et al.*, 1988) to readily evolve to persistent infections, the extended incubation times of HAV *in vivo* (i.e., up to 36 days), and the detection of DI RNAs in both these situations might be related phenomena.

8. *DI Particles and Viral Attenuated Vaccines*

Attenuated viral vaccines have classically been produced by serially passaging virus in nonnatural hosts or host cells. These multiple passages could have allowed the emergence of DI particles in at least some cases. Recently, measles-attenuated vaccine preparations have been shown to contain DI particles and/or subgenomic viral RNAs as well (Calain and Roux, 1988; Bellocq *et al.*, 1990). Whether these DI particles can replicate on vaccination and whether they participate in vaccine attenuation are open questions. Measles virus DI genomes were shown to survive 3 days in infected cells in the absence of helper virus before being rescued (Mottet *et al.*, 1990). In theory, this would allow vaccine DI genomes to be amplified in the body under conditions of low multiplicity of infection, through rescue by standard virus produced in adjacent cells. Likewise, the ability to detect rubella virus DI particles after three undiluted passages (Frey and Hemphill, 1988) makes likely the presence of DI particles in attenuated rubella vaccines (which are prepared following 27–80 passages).

9. *Toward More Sensitive DI Detection*

More sensitive detection of DI particles is likely to be needed to show their involvement in natural infections. This higher sensitivity might come from classical techniques such as the cytopathology inhibition

test of McLain *et al.* (1988), which appears to detect influenza DI particles with a 320,000-fold higher sensitivity than the previous infectious center inhibition test. PCR, however, would appear to be a method of choice to directly demonstrate the presence of defective viral genomes in specimens derived from natural infections. PCR may, however, suffer from its extreme sensitivity when attempting the detection of rare molecules or molecules with structures which are unfavorable for the amplification reaction. For example, amplification of copy-back RNA molecules has been found to be surprisingly difficult (P. Calain and L. Roux, unpublished observations). This is likely due to competition of the primers with the complementary RNA termini in the hybridization reactions (inter- versus intramolecular reactions). On the other hand, incorrect short-deletion DI DNAs have been amplified due to rare nonspecific priming when the appropriate DI RNAs were too long to be amplified (P. Calain and L. Roux, unpublished observations). Previous knowledge of the DI genome structures being sought may therefore be needed to exclude reverse transcriptase and Taq polymerase artifacts.

B. Retroviruses

Defective retroviruses have long been known to be potent biological modulators (Coffin, 1990) and, obviously, the most widely studied so far are the "acutely transforming defectives," which have replaced part of their genomes with an oncogene. They thereby induce cell-proliferative syndromes at high frequency in animals, and the basis for this increased pathogenicity is now reasonably well understood.

1. Feline Leukemia Virus (*FelV*)

Different classes of retroviruses have been shown to induce immunodeficiency diseases in various animal species (Desrosiers and Letvin, 1987), and this is rather common for *FelVs* (references cited by Overbraugh *et al.*, 1988). In viral DNA isolated from cat tissues infected with a natural isolate of *FelV* which consistently induces fatal immunodeficiency syndrome (*FelV*-FAIDS), variant forms of *FelV* genomes were identified and these became the predominant forms just prior to the onset of disease (Overbraugh *et al.*, 1988). By direct cloning from the infected tissues, a replication-defective variant (61C) was isolated and shown to be responsible for this increased pathogenicity. Variant 61C, in association with the common form (61E), induced the immunodeficiency syndrome, and transfer of the 3' end of the 61C genome (*env* long terminal repeat) onto the 61E genome conferred pathogenicity to the chimera. Amino acid substitutions, small dele-

tions and insertions in the gp70 coding region, as well as three nucleotide changes in the long terminal repeat were identified in 61C. Therefore, variant 61C, a replication-defective virus which can outgrow and interfere with its helper virus, appears to drastically modulate infections of cats.

2. Murine Leukemia Virus (MuLV)

Similarly, a replication-defective variant of MuLVs has been shown to be associated with severe immunodeficiency in mice (Chattopadhyay *et al.*, 1989) and to induce this syndrome (Aziz *et al.*, 1990). This variant, DUH5, was characterized by a deleted genome (4.8 kb versus 8.8kb for the helper virus) lacking most of the pol-env coding regions. The gag region, on the other hand, was relatively well conserved (Aziz *et al.*, 1990). DUH5 was shown to code *in vivo* for a 60-kDa protein representing a modified gag protein with a unique p12 domain (Chattopadhyay *et al.*, 1989; Huang and Jolicoeur, 1990). This Pr60^{gag} was normally myristylated, phosphorylated, and associated with the plasma membrane. However, it was not efficiently processed and could not be released into the medium in the absence of helper Pr65^{gag} (Huang and Jolicoeur, 1990). It appeared to interfere with efficient processing of Pr65^{gag}. DUH5 itself, without any detected helper function, was shown to induce immunodeficiency in correlation with oligoclonal proliferation of infected cells (Huang *et al.*, 1989). Immunodeficiency is thus postulated to derive from this primary neoplasia. These replication-defective interfering retroviruses were identified and found to be closely associated with immunodeficiency by *direct analysis* of infected tissues. This stresses the need for a similar strategy in searching for defective particles in virus infections in nature, because defective viruses are likely to be lost by usual methods of virus isolation.

3. HIV

A similar situation has not yet been described for human AIDS, caused by HIV. What is known, however, is that defective HIV viruses do exist in nature. For example, a highly defective strain with a non-functional tat protein was isolated from a healthy individual (Huet *et al.*, 1989). Comparison of HIV isolated in tissue culture from an individual at different times of infection, with endogenous viruses characterized by direct analysis at the time of isolation, showed marked discrepancies between the two populations of viruses (Myerhans *et al.*, 1989). This study, which was confined to the *tat* gene, inferred that many or most of the proviruses *in vivo* may be defective. This contrasted with a rather low frequency of inactivating mutations found in viral genome segments directly amplified and sequenced from pe-

ripheral blood mononuclear lymphocytes of HIV patients (Balfe *et al.*, 1990). Defective HIV viruses containing defects in *tat*, *rev*, or *gag* proteins have been experimentally produced (Gorelick *et al.*, 1990; Trono *et al.*, 1989; Green *et al.*, 1989; Malim *et al.*, 1989). These lethal variants expressed a dominant negative phenotype over the infectious virus and, although they are not DI viruses *stricto sensu*, they can interfere with efficient wild-type virus multiplication. The possible presence of such defective genomes in natural HIV infections is still open to question. Once again, such defective viruses would escape any detection method which involves preliminary amplification steps in tissue culture.

C. DNA Viruses

1. Epstein-Barr Virus (EBV)

EBV is a human herpesvirus that establishes a persistent replicative infection in oropharyngeal and genital sites. This contrasts with the stringently latent life cycle observed in circulating B lymphocytes. These different types of infections have been attributed to variations in host cell regulation. However, *in vitro* EBV replicative systems have been shown to contain a defective EBV form (see references cited by Patton *et al.*, 1990). This defective EBV contains a deleted rearranged DNA (het-DNA, for heterogeneous DNA) which appears to replicate preferentially over the standard EBV DNA (Miller *et al.*, 1985). When added to latently infected cells, het-DNA activates expression of endogenous EBV genomes and causes disruption of latency (Fresen *et al.*, 1978; Miller *et al.*, 1984). het-DNA can be transmitted horizontally from cell to cell, and therefore appears to be a defective form of EBV capable of modulating infections. PCR amplification of a sequence specific for the rearranged het-DNA has led to identification of this DNA segment in human epithelial lesions (oral hairy leukoplakia), known to contain abundant replicative EBV (Patton *et al.*, 1990). The possibility of participation of this defective form of EBV DNA in the modulation of natural human infections must therefore be considered.

2. Herpesvirus

Herpesvirus DI is known to promote establishment of persistent infections and to inhibit infected cell cytolysis (Frenkel, 1981). The interfering mechanisms are not understood. Competition of strong promoters present on the reiterated DI DNA with promoters of the helper virus DNA, leading to down-regulation of the helper functions

involved in cytolysis, has been proposed (Frenkel, 1981). More recently, a modified ORF was identified on an equine herpes type 1 DI genome. This modified ORF, created by rearrangement, results from the replacement of the last 97 amino acids, or an ORF of 469, by a sequence of 68 different residues (Gray *et al.*, 1989; Yalamanchili *et al.*, 1990). This has opened the possibility that this new function, if expressed by the DI genome, would be able to interfere with helper virus functions. It is tempting to speculate that the rearrangements involved in DI genome generation could create trans-dominant negative regulators of the type engineered by truncation of the transcriptional activator VP16 (see Section I; Friedman *et al.*, 1988).

3. *Hepadnavirus*

As discussed in Section I, a high incidence of defective HBV genomes containing a disruption of the precore coding sequence has been identified in patients having undergone anti-HBe seroconversion (Carman *et al.*, 1989). For three of these patients, exclusive appearance of such defective HBV genomes could be exactly correlated with seroconversion (Okamoto *et al.*, 1990). This was observed under conditions in which no genomes carrying defects in the precore region could be detected in three other patients who lacked anti-HBe antibodies (Okamoto *et al.*, 1990). Since anti-HBe seroconversion is generally tied to evolution toward chronic asymptomatic infections, it is tempting to speculate that the course of HBV infections is often modulated by defective viruses. A study by Miller *et al.* (1990), on the other hand, suggests that this may not be necessary. Infections of woodchucks with a serum pool containing defective particles and with a recombinant-derived virus pool (equivalent to a plaque-derived inoculum) led to 65% and 80% of chronic carriers, respectively, suggesting that defective virus in the inoculum is not essential for the establishment of persistent hepadnavirus infection (Miller *et al.*, 1990). These results were discussed in Section I.

D. *Satellite Virus (Delta Agent)*

Viroids, virusoids, and satellite viruses or satellite RNAs are commonly involved in plant diseases (see Section III). A thus far unique equivalent of such plant genomes in the reign of animal viruses is the human delta agent (reviewed by Taylor, 1990). Hepatitis delta virus (HDV) infection is closely associated with HBV because HDV uses HBV surface antigen to form enveloped particles capable of cell-to-cell transmission; hence, its definition as a satellite virus. HDV, however, is capable of autonomous RNA replication in the absence of HBV (Kuo

et al., 1991). Coinfection with HDV often results in exacerbation of the underlying HBV hepatitis (Rizzetto *et al.*, 1988). The molecular basis of this modulation of infection is not understood. Restricted levels of HBV replication have been reported in some cases, but high levels of both nucleic acids in the serum have been documented as well (Monjardino and Saldanha, 1990). HDV genome is transcribed into an mRNA which encodes the delta antigen (Hsieh *et al.*, 1990). Actually, two forms of the delta antigen have been detected in infected tissues, corresponding to two different RNA genomes with an ORF corresponding to proteins of 195 and 214 residues, respectively. The additional 19 amino acids consist of a carboxy-terminal extension of the small antigen resulting from a transition in the stop codon (Luo *et al.*, 1990). Remarkably, this variant genome accumulates on replication of the small protein-encoding genome. Whereas the small delta antigen strongly enhances genome replication, the large form acts as a dominant negative repressor of such replication (Chao *et al.*, 1990). Therefore, while the delta agent is itself a modulator of HBV infection, its own replication is, in turn, regulated by the generation of a defective mutant genome able to exert significant interference with the delta agent.

III. DI VIRUSES OF PLANTS

Plant viruses can be associated with a variety of small subviral RNAs such as satellites, DIs, and chimeric molecules with properties of both satellites and DIs. This section reviews our current knowledge of the replication and symptom modulation ability of these small RNAs, focusing primarily on systems in which both satellites and DIs have been found.

As summarized in Section I, satellites are defined as RNAs which require a helper virus for infectivity, yet share little sequence similarity with the viral genomic RNA(s) (Murant and Mayo, 1982). Satellites that encode structural proteins which compose the satellite capsid are referred to as satellite viruses, whereas satellites that are encapsidated in helper virus particles are called satellite RNAs. Satellites can be either linear or circular; circular satellites are commonly known as virusoids. One of the intriguing properties of satellites is their ability to modulate the symptoms of the helper virus, either by attenuating symptoms (analogous to many animal virus DIs, as described in Sections I and II) or by intensifying symptoms. The replication strategies of these molecules are also fascinating, since the helper viral replicase (which, presumably, is required for subviral RNA replication) must, in

many cases, recognize sequences and structures not present in the viral genome.

Only recently have DI RNAs been found to be associated with plant viruses. Two viruses shown to have DIs are members of the tombusvirus group: the cherry strain of tomato bushy stunt virus (TBSV, also known as petunia asteroid mosaic virus) (Hillman *et al.*, 1987) and cymbidium ringspot virus (CyRSV) (Burgyan *et al.*, 1989; Rubino *et al.*, 1990). A third virus associated with DI RNAs, turnip crinkle virus (TCV) (Li *et al.*, 1989), is a member of the structurally related carmoviruses. All three viruses support the replication of satellite RNAs. Both TBSV and CyRSV DIs ameliorate symptoms of the helper virus, while TCV DIs dramatically intensify viral symptoms on a variety of hosts. In this section we first discuss plant virus systems in which both DI and satellite RNAs have been well characterized, and then describe the effects of the subviral RNAs on replication and symptom production of their helper viruses. In Sections III, E and F we present information on the generation of DI and other discontinuous RNAs in plants and possible future uses of DIs in controlling disease.

A. TCV

TCV is a 30nm icosahedral plant virus with a single genomic RNA of 4051 nucleotides and a coat consisting of 180 subunits of a 38-kDa protein (Morris and Carrington, 1988). Although TCV is related to TBSV and CyRSV both structurally and at the nucleotide sequence level, the genomic organization of TCV differs markedly from the two tombusviruses; TBSV and CyRSV contain two additional ORFs beyond the coat protein ORF, and TCV has a small ORF upstream of the coat protein-coding sequence (Carrington *et al.*, 1989; Grieco *et al.*, 1989; Hearne *et al.*, 1990). TCV is associated with a variety of subviral RNAs including satellite RNAs, DI RNAs, and molecules with characteristics of both satellites and DIs (Fig. 1). All these subviral RNAs accumulate in such vast quantities that they are clearly visible in ethidium bromide-stained gels. Two isolates of TCV have been characterized: TCV-B and TCV-M. The TCV-B strain has mild symptoms on turnip and is naturally associated with a DI RNA, DI RNA G, and a satellite RNA, sat-RNA D (Li *et al.*, 1989). DI RNA G is a mosaic molecule composed of a sequence near the 5' end of TCV as well as viral terminal 3' -end sequences. The first 10 nucleotides at the 5' end of DI RNA G, however, are not derived from TCV genomic RNA, but rather share complete or nearly complete homology with the 5' ends of the TCV satellite RNAs. The next 10 nucleotides of DI RNA G are of unknown origin (or have diverged significantly from a genomic or satellite sequence).

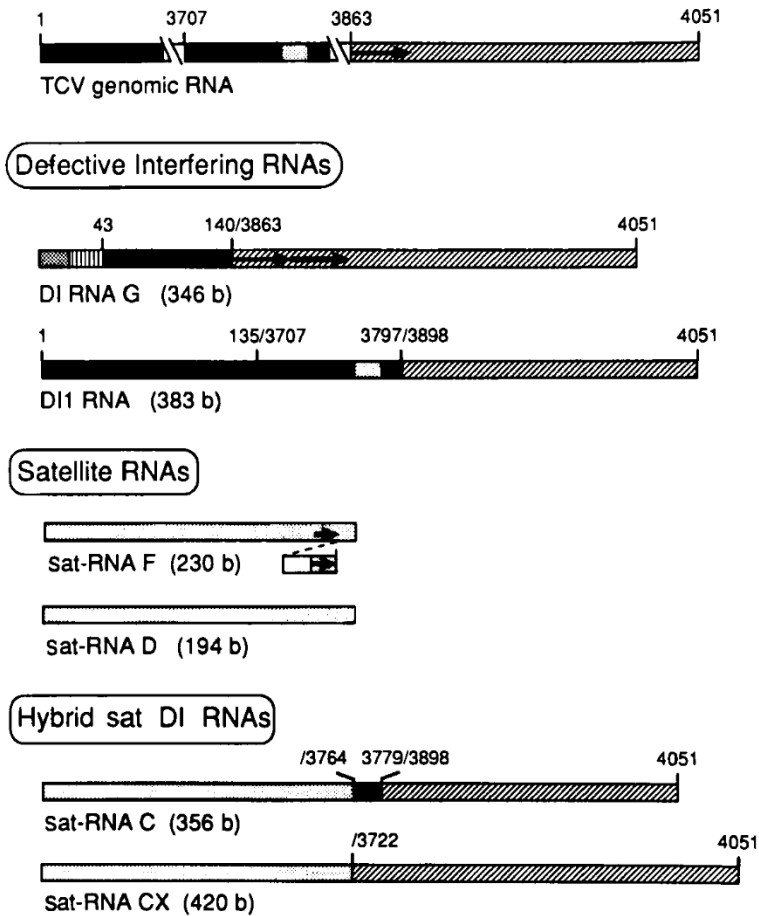


FIG. 1. Subviral RNA of turnip crinkle virus (TCV). Sequences in common are shaded alike.

A TCV-similar sequence begins with base 21 of DI RNA G, which corresponds to base 43 of TCV. Sequences of six DI RNA G cDNA clones reveal heterogeneity among numerous bases, single-nucleotide insertions and deletions, and a direct repeat of 36 bases (Li *et al.*, 1989).

TCV-B genomic RNA has been cloned and sequenced (Carrington *et al.*, 1989), and transcripts synthesized *in vitro* are infectious (Heaton *et al.*, 1989). Inoculation of turnip with *in vitro*-synthesized viral transcripts can result in the accumulation of DI RNAs *de novo* (Li *et al.*, 1989). Characterization of one *de novo*-generated DI RNA (DI1 RNA) revealed a molecule containing both the exact 5' and 3' ends of TCV flanking one interior segment.

The TCV-M isolate produces a severe disease on turnip and other hosts (Li and Simon, 1990) and is naturally found with two satellite RNAs, sat-RNAs D and F, and one RNA which is a hybrid between a satellite and DI RNA, sat-RNA C (Simon and Howell, 1986). sat-RNA C, which historically has been referred to as a satellite, has the complete sequence of sat-RNA D at the 5' end and two regions of TCV genomic RNA at the 3' end. Recently, a second hybrid satellite has been identified which contains a sat-RNA D sequence at the 5' end and a single segment of TCV genomic sequence at the 3' end (Zhang *et al.*, 1991).

All junctions of TCV discontinuous RNAs [DIs, hybrid satellite/DI RNAs and recombinant RNAs (see below)] have right-side junction sequences which can be classified as one of three dissimilar motifs of approximately 20 nucleotides each (Cascone *et al.*, 1990). One of the motifs is found near the 5' end of TCV (beginning at base 10), while a second is located at the extreme 5' end of the satellite RNAs and DI RNA G. The third motif, which is located at the right side of several junctions, is also located immediately downstream of the 5' end of the TCV subgenomic RNA which encodes the capsid polypeptide.

The satellite and DI RNAs naturally associated with TCV are efficiently packaged in the helper virus capsid (Altenbach and Howell, 1981; Heaton *et al.*, 1989). Using assays involving the protection of nucleic acid from RNase treatment by tightly bound coat protein, Wei *et al.* (1990) have identified several high-affinity coat protein binding sites on the RNA genome. None of these sequences, however, is present in the DI RNAs. Studies with purified DI RNA G suggest that an additional high-affinity site is located in the DI RNA sequence which is not protected in assays using genomic RNA. These authors propose that differential folding of the two molecules may occlude or expose this coat protein binding site. Both sat-RNA C and the DI RNAs also contain this coat protein-protected sequence, which may explain the efficient accumulation of these molecules *in planta*. Since sat-RNAs D and F lack sequence similarity with the helper virus, excluding 7 nucleotides at the 3' ends, and are efficiently packaged, other sequences with affinity for TCV coat protein remain to be discovered.

B. TBSV

TBSV, the type member of plant tombusviruses, is a 30-nm icosahedral virus consisting of 180 copies of a 41-kDa structural protein subunit in the capsid (Morris and Carrington, 1988). TBSV has a single-stranded positive-sense RNA genome of 4776 nucleotides (Hearne *et*

al., 1990) and can support the replication of a 0.7-kb satellite RNA which was originally isolated from plants infected with CyRSV (Hillman, 1986). A 0.4-kb DI RNA associated with a laboratory stock of TBSV markedly attenuates the symptoms expressed by the virus on tobacco [*Nicotiana clevelandii* (Hillman *et al.*, 1987)]. The 396-base RNA is a complex mosaic molecule composed of five noncontiguous segments of viral homology. Three nucleotides at the extreme 5' terminus of this DI RNA differ from the viral 5'-end sequence, and homology begins 13 nucleotides into the genomic RNA sequence. Computer analysis of individual junctions reveals no obvious sequence or structural similarities. Unlike the satellite RNA, which is efficiently encapsidated by helper virus encoded coat protein, the DI RNA is inefficiently packaged, composing less than 30% of the total encapsidated RNA on a molar basis (Morris and Hillman, 1989). DI RNAs have also been generated following high-multiplicity passaging of a clonally pure TBSV inoculum (Morris and Hillman, 1989). These DI RNAs, presumably generated *de novo*, vary in size (< 0.7 kb) and degree of symptom attenuation.

C. CyRSV

CyRSV contains a single-stranded positive-sense genomic RNA of 4733 bases and shares nearly 70% sequence similarity with TBSV (Grieco *et al.*, 1989; Hearne *et al.*, 1990). Isolates of CyRSV can contain either a 621-base satellite RNA or a 499-base DI RNA (Burgyan *et al.*, 1989; Rubino *et al.*, 1990). Approximately 17% of the satellite primary sequence is similar to several interspersed regions of CyRSV genomic RNA. The mosaic DI RNA is composed of six discontinuous fragments of CyRSV genomic sequence. Unlike the DI RNA of TBSV and DI RNA G of TCV, CyRSV DI begins at the 5' end of the helper virus genomic RNA. The right sides of the junction sequences share some sequence similarity with the very 5' end of the genomic RNA. Unlike the satellite RNA, CyRSV DI RNA is not efficiently packaged, which suggests that common sequences between the satellite and the genomic RNA, not present in the DI RNA, may be candidates for encapsidation signals (Burgyan *et al.*, 1989).

Full-length transcripts synthesized *in vitro* from cloned CyRSV genomic RNA are infectious (Burgyan *et al.*, 1990). DI RNAs were found to accumulate in *N. clevelandii* previously inoculated with sap from CyRSV transcript-infected plants (Burgyan *et al.*, 1991). The *de novo*-generated DI RNAs were similar to the DI RNA in the original inoculum in having sequences entirely derived from the helper virus genome. RNA samples taken from younger and older leaves contained

different DI RNA sizes, with larger DIs present in older tissue. Sequence analysis revealed that the smaller DIs, which were stable through later passages, were nearly always completely contained within the larger molecules.

*D. Attenuation and Intensification of Symptoms
by Plant Subviral RNAs*

DI RNAs associated with TBSV and CyRSV dramatically attenuate the symptoms of their helper viruses. Inoculation of *N. clevelandii* with TBSV genomic RNA purified over a sucrose gradient results in severe systemic necrosis and plant death within 2 weeks (Hillman *et al.*, 1987). Plants infected with an inoculum containing purified virus and DI RNA exhibit local necrotic lesions only on the inoculated leaves followed by a persistent infection. Symptom attenuation is correlated with both a decrease in the amount of virus recovered from plants and an increase in the accumulation of DI RNA. Studies using tobacco protoplasts to analyze the influence of a 0.7-kb DI RNA on TBSV replication indicate that the presence of the DI results in a 65% reduction in the rate of helper virus genomic RNA synthesis (Jones *et al.*, 1990). By analyzing the rate of genomic RNA synthesis, Jones *et al.* were able to discount the possibility that the DI RNA affects viral accumulation due to enhanced degradation of the genomic RNA or selective suppression of subgenomic RNA synthesis. The 0.7-kb CyRSV satellite also has an effect on symptoms produced by TBSV. When the satellite is inoculated with TBSV on *N. clevelandii*, symptoms are slightly less severe and delayed by about 1 week (Hillman, 1986). The level of accumulation of viral genomic RNA in the presence of the satellite is substantially reduced in whole plants.

The satellite and DI RNAs associated with CyRSV also ameliorate the symptoms of the virus on *N. clevelandii* (Burgyan *et al.*, 1989). Plants inoculated with genomic RNA alone exhibit apical necrosis followed by plant death. Infection of plants with viral genomic and satellite RNAs reduces symptoms to stunting with no necrosis. Plants infected with CyRSV and the DI RNA produce mild symptoms visible only on younger leaves. Although the effect of DI or satellite RNA on virus levels has not been rigorously examined, CyRSV genomic RNA levels are decreased in plants infected with both genomic and DI RNAs (Burgyan *et al.*, 1989).

Unlike DI RNAs of other plant or animal viruses, both the hybrid satellite/DI and DI RNAs of TCV markedly intensify the symptoms of the helper virus (Altenbach and Howell, 1981; Li *et al.*, 1989). Most host plants infected with helper virus with or without the two small

satellites (sat-RNAs D and F) exhibit mild symptoms such as stunting, slight leaf crinkling, and mosaic coloring (Simon *et al.*, 1989). Addition of either sat-RNA C or DI RNA G results in stunting and tightly crinkled leaves with a dark green pigment (Li *et al.*, 1989; Li and Simon, 1990). For one plant, *Arabidopsis thaliana*, addition of sat-RNA C results in the death of most ecotypes (Li and Simon, 1990; X. H. Li and A. E. Simon, unpublished observations). Curiously, symptoms are intensified even though sat-RNA C and DI RNA G interfere with the accumulation of TCV (Li *et al.*, 1989; A. E. Simon, unpublished observations). Virus levels are 5-fold lower when DI RNA G is included in the inoculum and about 2-fold lower in the presence of sat-RNA C.

All the TCV subviral RNAs which increase symptoms have 153 bases of genomic RNA 3'-end sequence in common (see Fig. 1). Studies using cloned transcripts of sat-RNA C have indicated that this region is indeed responsible for symptom production (Simon *et al.*, 1988). Attempts to define precise sequences responsible for increased symptoms have been unsuccessful due to the limited infectivity of sat-RNA C (Simon *et al.*, 1988; C. D. Carpenter and A. E. Simon, unpublished observations) or DI RNA G (Li and Simon, 1991) transcripts with mutations near the 3' end.

The ability of subviral RNAs to produce disease symptoms is best understood for the satellite RNA associated with cucumber mosaic virus (CuMV); alterations at any of three nucleotides distinguishes necrogenic and nonnecrogenic satellites (Devic *et al.*, 1990; Sleat and Palukaitis, 1990b). Computer-generated secondary-structure predictions for necrogenic and nonnecrogenic satellites do not reveal any correlations between structure and pathogenesis (Sleat and Palakaitis, 1990b), implying that the primary nucleotide sequence is somehow responsible for disease symptoms. Furthermore, the secondary-structure model for one necrogenic satellite indicates that the necrogenic domain is in a highly nuclease-sensitive region (Hidaka *et al.*, 1988), suggesting that this region of the satellite may interact with either host- or virus-encoded factors to initiate disease production.

The intensification of symptoms by the virulent satellite or DI RNA of TCV is completely dependent on the origin of the helper virus genomic RNA (C. D. Carpenter, X. H. Li, and A. E. Simon, unpublished observations) and the ability of the genomic RNA to produce symptoms on a host plant (Li and Simon, 1990). Plants display increased symptoms only if inoculated with subviral RNA and the genomic RNA from the TCV-M isolate. Similar results have been obtained for several CuMV satellites; systemic chlorotic symptoms are produced on tobacco only when the satellites are associated with one subgroup of the CuMV helper virus (Sleat and Palukaitis, 1990a). These results sug-

gest that an interaction between satellite RNAs or DI RNAs and the viral genomic RNA or encoded product is necessary for symptom production.

E. Generation of Plant DI RNAs

A template-switching model in which specific sequences are targeted by the replicase during reinitiation of synthesis has been proposed for the generation of DI RNAs and the satellite/DI hybrid RNAs in the TCV system (Cascone *et al.*, 1990). This model is based on the similarity among right-side junction sequences and one of three motifs (Cascone *et al.*, 1990; Zhang *et al.*, 1991). Since the motifs are also found at the 5' ends of the TCV genomic RNA and subviral RNAs as well as near the initiation site of the 1.45-kb subgenomic RNA, template switching by the replicase has been suggested to occur during replication of minus-strand RNA. This model is similar to one proposed by Re *et al.* (1985) for the generation of Sendai virus DI RNAs. Pyrimidine-rich putative promoter signals are found at one side of Sendai virus DI junctions which resemble a sequence located 5–16 nucleotides from the 3' end of the genomic RNA. A similar mechanism may also be responsible for the generation of CyRSV DI RNAs, since there is sequence similarity at the right side of all junctions (Rubino *et al.*, 1990). Recently, recombination between TCV satellites, and between satellites and the genomic RNA, has been detected (Cascone *et al.*, 1990; Zhang *et al.*, 1991). The right-side junction sequences of the single and double recombinant RNAs are all similar to one of the three motifs mentioned above. The importance of specific nucleotides within one motif was recently demonstrated by the elimination of recombination between satellite RNAs following single-base alterations within the motif present in sat-RNA C, which is always found at the right side of satellite recombinant junctions (P. J. Cascone and A. E. Simon, unpublished observations).

After initial generation of a DI RNA, further accumulation depends on the ability of the molecule to be replicated and to move along with the helper virus through the plant. The observation that most subviral RNAs of TCV range in size from 346 to 420 bases indicates that the size of the DI RNA may play an important role in efficient accumulation. Further, more conclusive evidence for the importance of size comes from recent studies using an infectious clone of TCV DI RNA G (Li and Simon, 1991). Plants inoculated with transcripts of the DI containing 30 base deletions near the 5' end do not accumulate any detectable DI RNA. However, if the deleted sequence is replaced with a similar-sized fragment derived from a bacterial plasmid, the DI RNA

accumulates at normal levels. Replacement of the 30-base deletion with 60 unrelated nucleotides also produces a viable molecule. There are at least two possible explanations for the size requirement of the DI RNA: (1) Length of the molecule may be important in encapsidation into the TCV isocahedral capsid; and (2) distance between the 3' and 5' ends may play a role in replication processes. Further work using plant protoplasts which are competent to replicate the viral and subviral RNAs should distinguish between these two possibilities.

F. Future Uses of Plant DI RNAs

Viruses are responsible for major crop losses worldwide, and ways of improving plant resistance to viruses are currently being pursued in a number of laboratories. Since DI RNAs (in general) interfere with virus accumulation and disease production, DI RNAs associated with viruses are actively being sought. However, with the exception of the plant rhabdovirus *Sonchus* yellow net virus (Ismail and Milner, 1988) and the reovirus wound tumor virus (Anzola *et al.*, 1987), DI RNAs have been found only for the related viruses described above. The creation of artificial DI RNAs may be a viable alternative to reliance on naturally formed DIs. Although these artificial DIs may not be packaged for transport through the plant, possibly due to a lack of packaging signals and/or size constraints, the ability to transform plants such that each cell produces DI RNA transcripts should alleviate the need for natural movement. The DIs would still require the *cis*-acting signals necessary for replication by the helper virus, and identification of such replication signals is an active area of plant virus research. Similar experiments using satellite RNAs to control the pathogenesis of CuMV proved quite successful; tobacco transformed with cDNA to a CuMV satellite which ameliorates CuMV symptoms, under transcriptional control of a constitutive plant promoter, produces low levels of satellite RNA which are greatly amplified following infection with CuMV. This results in disease attenuation (Harrison *et al.*, 1987).

IV. SUMMARY

DI viruses and defective viruses generally are widespread in nature. Laboratory studies show that they can sometimes exert powerful disease-modulating effects (either attenuation or intensification of symptoms). Their role in nature remains largely unexplored, despite recent suggestive evidence for their importance in a number of systems.

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