Origin and Replication of Defective Interfering Particles

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1 Introduction

Defective interfering virus (DI) particles represent a major controlling element of virus replication. They are constantly generated at low levels by infectious virus and only amplify to interfering levels when the parent helper virus is abundant. This autointerference phenomenon, as it was called when first discovered, is achieved by rearrangements and deletions of the standard virus genome such that the resulting "incomplete form" of the virus can preferentially replicate.

Studies on all aspects of DI have greatly expanded in the last decade. This interest stems not only from their characteristic attenuating properties but from their role in the establishment and maintenance of persistent viral infections. I shall limit myself in this review mostly to a discussion of recent findings regarding DI of RNA viruses. At the present time much less is known about the properties and biological importance of DI of DNA viruses (see review by *Huang* and *Baltimore* 1977) and except for a brief overview of their current status (Section 6) they will not be discussed in detail.

It is generally agreed that RNA viruses (with the exception of retroviruses) lack the ability to integrate double-stranded DNA copies or fragments of their genome into eucaryotic host cell chromosomes (see discussion Section 4.2). RNA viruses may therefore rely on alternative mechanisms of attenuation, such as DI, to achieve long term virus-host cell association. An additional important facet of these DI is that their generation apparently involves recombination directly at the RNA level. The relationship, if any, between this type of genomic rearrangement and other recombinational events in RNA viruses is of obvious importance. Furthermore, the possible mechanistic and/or evolutionary relationship between RNA recombination in viruses and gene splicing in eucaryotic mRNAs is intriguing.

This review will first introduce the reader to general aspects of DI structure and function, followed by a detailed survey of our current information regarding genome sequence rearrangements in DI of RNA viruses. The remainder of the article will attempt to integrate this information within a framework of hypotheses and models to account for the origin and preferential replication of DI RNAs. We will omit from discussion the retroviruses and the plant RNA viruses because of difficulties in defining DI operationally in these groups. Multiple interactions between endogenous and exogenous, defective, and nondefective retroviruses often obscure a straightforward DI phenomenon, although some reports lend support to such a concept (*Huang* and *Baltimore* 1977; *Estis* and *Temin* 1979; *Canaani* and *Aaronson* 1980). Plant RNA viruses on the other hand are often multipartite systems with two or three distinct particles, all of which are necessary for infectivity. In addition, until the recent advent of protoplast cultures, simple direct tests of interference by purified particles in synchronously infected cells have been impossible. Nevertheless, some reports of defective plant viruses are consistent with their being called DI (see *Huang* 1973; *Atabekov* 1977) and future studies may well uncover a more common occurrence.

Several reviews on the subject of DI have been published in recent years. For a general comprehensive description of DI (both RNA and DNA) and their properties the reader is referred to *Huang* and *Baltimore* (1977). The more recent review of *Holland* et al. (1980) discusses both the structure and biological properties of DI and focuses on their role in virus persistence. Detailed reviews of DI in particular virus groups have also appeared: poliovirus, *Cole* (1975); rhabdoviruses, *Reichmann* and *Schnitzlein* (1979); to-

gaviruses, *Stollar* (1979, 1980); arenaviruses, *Pedersen* (1979); influenza, *Nayak* (1980); and virus-like particles of yeast (*Bruenn* 1980). Short review articles on selected properties of vesicular stomatitis virus (VSV) DI are also included in the CRC volume series on Rhabdoviruses (*Faulkner* and *Lazzarini* 1980; *Reichmann* and *Schnitzlein* 1980; *Kang* 1980).

2 General Aspects of Defective Interfering (DI) Particles

2.1 Ubiquity of DI in RNA Viruses

Table 1 presents an updated list of RNA viruses reported to generate DI. It is obvious from this table that all serious attempts to generate DI in any RNA virus group

Table 1. Occurrence of DI in RNA Viruses

Virus group	Member name	References[a]
Negative strand		
Rhabdo	Vesicular stomatitis, rabies, bovine ephemeral fever, others	*Reichmann* and *Schniztlein* (1979)
Paramyxo	Sendai	*Kolakofsky* (1979)
	Newcastle disease	*Roman* and *Simon* (1976)
	Measles	*Rima* et al. (1977)
	Mumps	*Norval* (1979)
Orthomyxo	Influenza, fowl plague	*Nayak* (1980)
Arena	Lymphocytic choriomeningitis, Amapari, Parana, Pichinde	*Pedersen* (1979)
	Tacaribe	*Gimenez* and *Compans* (1980)
Bunya	Bunyamvera	*Kascsak* and *Lyon* (1978)
	LaCrosse	*Bishop* and *Shope* (1979)
	Turlock	*Schnurr* and *Hardy* (1980)
Positive strand		
Picorna	Poliovirus	*Lundquist* et al. (1979)
	Mengovirus	*McClure* et al. (1980)
Calici	Feline calicivirus	*Schaffer* (1979)
Toga	Sindbis, Semliki Forest, West Nile	*Stollar* (1979)
	Japanese encephalitis	*Schmaljohn* and *Blair* (1977)
Corona	Mouse hepatitis	*Robb* and *Bond* (1979)
Double strand		
Reo	Reovirus	*Ahmed* and *Fields* (1981)
Orbi	Bluetongue virus of sheep	*Verwoerd* et al. (1979)
Fungal	Yeast-killer, others	*Bruenn* (1980)
Other	Infectious pancreatic necrosis virus	*MacDonald* and *Yamamoto* (1978)

[a] The references listed are either recent review articles or publications wherein earlier work on the particular DI is described. For a list of the earliest reports describing DI in various virusgroups the reader is referred to *Huang* and *Baltimore* (1977)

(retroviruses not considered) have so far been successful. No exceptions have yet been reported and it therefore appears to be a general rule.

The methods employed for detecting and purifying DI have been reviewed recently (*Huang* and *Baltimore* 1977) and will not be detailed here. In general, biological cloning of a virus leads to the disappearance of most or all DI in a given working stock. During passage at high multiplicities of infection any newly arising or preexisting DI particle replicates because of complementation by helper virus. The population of virions eventually becomes mostly DI because the latter replicate preferentially and inhibit the growth of the standard virus.

The ease with which DI are produced varies widely in different virus groups. For example, high titer cloned stocks of VSV and influenza virus can produce large amounts of DI in as little as two or three undiluted passages. In contrast, at least 20 or so serial passages are needed to detect significant amounts of poliovirus DI. There is no simple way to predict the ease with which a particular RNA virus will produce DI since many different factors are involved. These include growth conditions, multiplicities of infection, the host cell, relative rates of standard virus replication and DI enrichment, virus strain differences, and intrinsic rates of DI generation.

2.2 Biological and Structural Properties

As defined by *Huang* and *Baltimore* (1970, 1977) the criteria for the definition of DI are as follows: 1. Antigenic similarity to standard virus, 2. requirement for homologous parent virus as helper for replication, 3. preferential replication of DI over standard virus (also called enrichment), and 4. interference with the replication of the standard virus. So far, all DI examined critically contain deletions in a part of the genome essential for infectivity. However, as pointed out before (*Huang* and *Baltimore* 1977) it is possible to imagine point mutations which might fall within the limits of these criteria.

The structural similarity between standard virus and DI extends to overall shape and size in viruses with icosahedral symmetry such as picornaviruses and reoviruses. In most of these cases, DI contain less total RNA than the parent virus and can sometimes be separated by density gradient centrifugation. In VSV and other rhabdoviruses, the length of the particle RNA governs the overall size of the bullet-shaped viruses, and since DI contain shorter RNAs they are easily separated by size from the standard virus. In more pleiomorphic viruses, such as influenza and measles virus, DI tend to be smaller than the standard particle but the variability in size of the virion population as a whole makes it difficult to achieve clean separation (for a discussion of DI sizes and sedimentation properties, see *Huang* and *Baltimore* 1977).

In general, the requirement for helper virus as well as interfering activity is limited to the homologous parent or to serologically related strains. The nature of the helper function supplied by the standard virus varies in different virus systems. For example, the VSV standard virus almost always provides all five virus-coded proteins for replication and encapsidation of a DI RNA template which does not code for any translation products. In poliovirus, however, the standard virus helper, at least in some cases, provides the missing capsid proteins to a DI RNA which gives rise to all other virus translation products (see Section 5.2). The enrichment and interfering properties of DI also vary in different virus systems, but these are subject to important modulations by the host cell. These effects are described in more detail below (Sections 4.1 and 5.1).

Since DI are not antigenically distinct from their parent virus, their biological properties are attributable to the genome deletions they contain. DI interference with standard genome replication is an intracellular phenomenon occurring after virus penetration but generally early during the growth cycle. It is not mediated by interferon or other host-cell coded products, and is distinct from the heterologous type of interference often observed when two different viruses infect the same host cell. There is no evidence, so far, indicating that an aberrant virus-coded translation product is reponsible for the interfering or preferential replication properties of DI. In the best studied systems, it appears that some property of the DI template RNA itself leads to an altered interaction with the polymerase activities replicating the viral polynucleotides.

2.3 Role of DI in RNA Virus Persistence

This topic which has recently been reviewed in detail (*Holland* et al. 1980) is briefly discussed here because it underlines the importance of understanding the mode of action of DI. The pioneering studies of *Holland* and *Villarreal* (1974) provided the first clear evidence for a DI requirement in establishing stable VSV carrier cell cultures in vitro. Since then, *Holland* and colleagues, as well as many other laboratories, employing several different RNA viruses, have also shown a requirement for DI in the establishment and/or maintenance of persistent infections in cell culture. Other factors, such as interferon and ts virus mutants, are also likely to play a role but it is clear that the attenuating properties of DI, either alone or in conjunction with these other factors, allow host cells to survive an otherwise cytocidal infection.

Two important facts regarding DI and virus persistence need be mentioned here. First, the overall structure and apparent biological activity of DI recovered from persistently infected cell cultures or animals appear very similar if not identical to DI generated by high multiplicity passages in permissive cell lines (*Holland* et al. 1980). Second, DI appear to act as a selection pressure for rapid and extensive mutational drift of standard virus during long term persistence (*Holland* et al. 1979; *Wechsler* et al. 1979). This is exemplified by the rapid emergence of standard virus mutants which are no longer subject to interference by DI originally employed to establish a persistently infected culture. The new standard virus, however, is still capable of generating new DI which interfere with its own growth (see Section 5.4.2). This important phenomenon has now been observed in several different virus systems: rabies virus (*Kawai* and *Matsumoto* 1977); lymphocytic choriomeningitis virus (*Jacobson* et al. 1979); VSV (*Horodyski* and *Holland* 1980); Sindbis virus (*Weiss* and *Schlesinger* 1981); and, at least in part, the "killer" virus-like particles of yeast (*Kane* et al. 1979; *Bruenn* and *Brennan* 1980). This selective effect of DI on the evolution of RNA viruses in culture may yet harbor an even more important role for these particles in nature.

3 Genome Rearrangements in DI RNAs

3.1 Overview

Single-strand RNA viruses of animals are classified as positive-strand if the encapsidated RNA molecule is infectious by itself, i.e., corresponds to a functional mRNA molecule.

Negative-strand viruses on the other hand carry the opposite strand, and are strictly dependent on viral transcriptase enzymes packaged within the virions to initiate the infectious cycle (*Baltimore* 1971). Similarly, double-stranded RNA viruses also depend on a particle-bound transcriptase for infectivity (*Shatkin* and *Sipe* 1968).

Many of our current concepts regarding the origin and replication of DI are derived from the VSV system which has been studied in most detail. This rhabdovirus serves as a model for negative-strand viruses in general, and in particular for paramyxoviruses which employ a very similar if not identical strategy for replication. DI of the paramyxovirus Sendai closely resemble VSV DI in their genome structure (see below). No details of genomic sequence rearrangements in DI are known for other important rhabdoviruses, such as rabies or paramyxoviruses, or for measles, mumps, and Newcastle disease virus.

The only other negative-strand virus group where DI have been characterized in some detail at the molecular level is the orthomyxovirus (influenza) group. Current cloning and sequencing studies of the eight virion RNA segments of the influenza genome are paving the way for rapid progress in the characterization of DI RNAs generated in this group.

Biological studies of arenavirus DI have been carried out for more than a decade but little is yet known about the nature of genome sequences in their RNAs (the standard genome consists of two viral RNA segments). This virus group shows a propensity for establishing persistent infections both in cell culture and in the animal. Lymphocytic choriomeningitis virus (LCM) causes life long infections in mice, and evidence for DI involvement in this natural disease, as well as in persistently infected cell cultures, is strong (reviewed by *Pederson* 1979, and *Holland* et al. 1980).

The remaining negative-strand virus group is a relatively newly defined and large family of arboviruses, the Bunyaviruses, and again detailed characterization of DI RNA species is lacking (the standard genome consists of three segments). An important aspect of this group (also shared by most togaviruses and some rhabdoviruses and reoviruses) is the ability to grow both in vertebrate and invertebrate host cells. The possible role of DI in the natural transmission cycle of arboviruses is yet to be explored.

In the positive-strand RNA virus groups, both togavirus and picornavirus DI have been studied in some detail. Except for one preliminary report (see Table 1) on the occurrence of DI in the newly defined caliciviruses, nothing else is known regarding DI in this group. The remaining positive-strand virus family, the coronaviruses, are just beginning to attract the attention of molecular virologists and no definitive identification of DI have yet been reported.

Among the double-strand RNA viruses, the best studied DI system is the yeast killer virus (see review by *Bruenn* 1980), although the realization that these viruses generate DI has only surfaced recently (Table 1). Reovirus DI were discovered relatively early but they have not been characterized extensively. The salmonid infectious pancreatic necrosis virus (IPNV), which contains two double-strand RNA segments, is also known to generate DI RNAs which have not been characterized at the molecular level. In both reovirus and IPNV, DI do appear to be involved in persistence (*Holland* et al. 1980; *MacDonald* and *Kennedy* 1979).

3.2 Vesicular Stomatitis Virus (VSV) DI RNAs

3.2.1 General Properties and Types of Molecules

Several laboratories have contributed to determining the size, cistronic origin, and structure of VSV DI RNAs. I will mention here the main conclusions from these earlier studies (for details, see review by *Reichmann* and *Schnitzlein* 1979) and discuss the more recent work on the recombinant sites in these DI RNAs.

VSV DI RNAs range from approximately 10 to 60% of the standard VSV genome size which is about 11 kb long. Each DI particle RNA is packaged as a single molecule within a helical ribonucleoprotein the length of which is determined by the size of the RNA. This structural feature, as mentioned before, is responsible for the ease with which the shorter truncated particles can be purified free from standard virus. In general, a given clonal isolate of standard virus gives rise to one or a few major DI species after a few passages at high multiplicities of infection. A DI band of a particular size can be isolated directly from a sucrose velocity sedimentation gradient, and amplified in a second cycle of infection with DI-free standard virus.

A large number of independently isolated VSV DI have been studied in various laboratories over the last decade. A uniform system of nomenclature has recently been proposed to clarify the relationship between the different isolates (*Reichmann* et al. 1980). As a result, VSV DI previously known by names such as tsG31 ST (ts = temperature sensitive, G = Glasgow isolate of Indiana VSV strain, 31 = mutant number designation of group III complementation group, ST = short truncated or T particle) have now been renamed VSI tsG31 *DI 0.10* (5′, 65%), where VSI stands for VSV Indiana strain, tsG31 as above, *0.10* for the fractional length of the DI RNA molecule in comparison to the standard genome, and (5′, 65%) for derivation from the 5′-end of the genome and percentage level of self-annealing. This system has not yet been adopted widely, and may in some cases lead to confusion (such as more than one new designation for the same isolate passed on to different laboratories). To avoid ambiguity in this review, I will refer to particular isolates by both old and new names (if the latter is already in use in the literature) and point out particular situations where uncertainties exist.

In contrast to standard virus, VSV DI sometimes package mixtures of minus- and plus-strand polarity RNAs (*Roy* et al. 1973; *Perrault* and *Leavitt* 1977a). The minus strands are apparently always in excess except in the case of the so-called snap-back DI RNAs where the plus and minus strands are covalently-linked as a hairpin molecule (*Lazzarini* et al. 1975; *Perrault* 1976; *Perrault* and *Leavitt* 1977a). Why VSV DI sometimes package some plus strands in addition to minus strands is not entirely clear but it is likely to be related to the structural features at the ends of their RNAs (see Section 3.2.2).

All VSV DI RNAs examined so far contain virus-coded nucleotide sequences only. At least five different structural types of molecules have been reported to date (Fig. 1). In addition, these fall into two classes on the basis of transcriptional ability (*Perrault* and *Semler* 1979; see also Section 5.2).

The standard VSV genome is transcribed both in vivo and in vitro (catalyzed by the endogenous virion polymerase) into five monocistronic mRNAs, each coding for one of the five virus proteins (see Section 5.2 for a more detailed discussion of VSV transcription). The base sequence of all of the noncoding and a large proportion of the coding

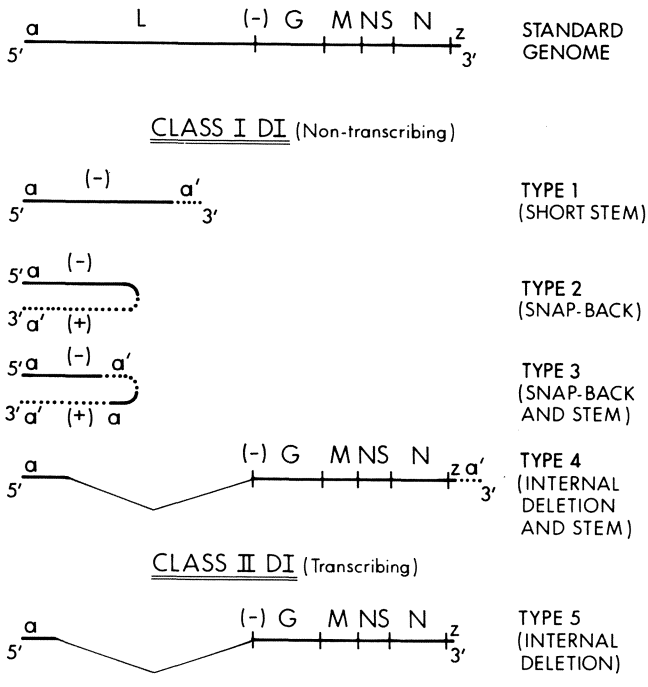

Fig. 1. Types of genomic sequence rearrangements in VSV DI. The standard virus minus-strand genome (about 11 kb), coding for five genes (L, G, M, NS, and N), is illustrated at the top of the figure. Short stretches of genome terminal sequences, 45–70 nucleotides at the 5′-end and 50 nucleotides at the 3′-end, are represented by the letters 'a' and 'z' respectively. Dotted lines indicate plus-strand sequences and a′ stands for a complementary copy of sequence a. The light solid lines in types 4 and 5 show deleted regions of the genome

regions are now known (*Keene* et al. 1978; *McGeoch* and *Turnbull* 1978; *Semler* et al. 1979; *Rowlands* 1979; *McGeoch* and *Dolan* 1979; *McGeoch* 1979; *Rose* and *Iverson* 1979; *Keene* et al. 1980; *Schubert* et al. 1980; *Rose* 1980; *Rose* et al. 1980a, b). The replication of the genome involves the synthesis of a full-size plus strand which then serves as template for minus-strand replication *(Wagner* 1975).

A variety of studies employing different approaches have shown that the majority of VSV DI RNAs (class I, types 1–3 in Fig. 1) contain a contiguous segment from the 5′-end of the standard genome. The evidence for this comes from hybridization to individual VSV mRNAs (*Leamnson* and *Reichmann* 1974; *Stamminger* and *Lazzarini* 1974; *Schnitzlein* and *Reichmann* 1976; *Schnitzlein* and *Reichmann* 1977a) and comparative oligonucleotide maps, which show that the smaller DI RNAs are subsets of the larger ones and correspond to sequences within the L cistron (*Clewly* et al. 1977; *Kang* et al. 1978a; *Clewly* and *Bishop* 1978; *Clerx-Van Haaster* et al. 1980). In addition, the presence of the exact 5′-terminal sequence of the standard genome in various DI RNAs was established through analysis of terminal complementarity and base sequencing studies described in the following section.

3.2.2 Terminal Sequence Complementarity

The hallmark of the most abundant class of VSV DI RNAs (class I, Fig. 1) is the presence of inverted complementary terminal sequences not found in the standard genome. The first evidence for this structural feature came from electron microscopic observations of circular DI RNAs with characteristic small panhandles or stems in VSV (*Perrault* 1976; *Perrault* and *Leavitt* 1977b) and in the paramyxovirus Sendai (*Kolakofsky* 1976). Isolation of the complementary VSV stem sequences from a short VSV DI (tsG31 ST, referred to as *DI 0.10* in the new nomenclature), following digestion of the single-stranded portion of the molecules with ribonuclease, yields a uniform size duplex molecule originally estimated to be approximately 60 bp in length (*Perrault* and *Leavitt* 1977b), and now known to be 53 bp long (*S.T. Nichol* and *J. Perrault* unpublished). By labeling the 5′-ends of these duplex stem molecules, or the 5′-end of the standard genome RNA, and using these probes in various hybridization experiments with unlabeled preparations of the same RNAs or other VSV DI RNAs the following conclusions were reached: 1. the 5′-end sequence of the standard genome and the DI RNAs are identical for the length of the duplex stems; 2. the 3′-end sequence of the DI RNAs (one strand of the duplex stem molecules) is not present within the minus strand of the standard genome; 3. this short extragenomic sequence at the 3′-ends of DI RNAs corresponds to the 3′-end of the complementary plus strand of the standard genome (*Perrault* et al. 1978). The extragenomic origin of these sequences was also suggested in other studies by the appearance of two unique oligonucleotides in T1 ribonuclease-digested DI RNAs, one of which appears to be derived from the 3′-end since it binds to a borate-affinity column (*Freeman* et al. 1978).

Several studies determining the base sequence corresponding to the ends of the VSV genome and its DI RNAs have confirmed the validity of the above conclusions. These include: 1. direct RNA sequencing of endogenous VSV particle polymerase products (see Section 5.2), which represent complementary copies of the 3′-end of DI RNAs (*Semler* et al. 1978; *Schubert* et al. 1978), or the 3′-end of the standard genome (*Colonno* and *Banerjee* 1978a); 2. direct RNA sequencing of the 3′-end of the standard genome and a number of DI RNAs (*Keene* et al. 1978; *Keene* et al. 1980); 3. cDNA sequencing of the 3′-end of the standard genome (*McGeoch* and *Dolan* 1979; *Rowlands* 1979); 4. direct RNA sequencing of the 5′-ends of the DI RNAs (*Schubert* et al. 1979; *Schubert* et al. 1980) and of the standard genome (*Semler* et al. 1979). The results from these sequencing studies are summarized in Figure 2. The standard genome of the VSV Indiana serotype shows a limited terminal sequence complementarity (14 out of the first 17 terminal residues). This limited complementarity undoubtedly accounts for the inability to isolate stable double-stranded stem structures from the standard VSV Indiana RNA. The terminal sequences of the VSV Indiana DI RNAs examined so far (excluding snap-back molecules discussed below) are capable of forming a unique size stable duplex varying from 45 to 70 bp in different isolates. A similar situation presumably holds true for the New Jersey VSV strains and some of its DI RNAs but in this case the limited terminal complementarity of the standard genome extends to the first 20 bases and gives rise to a stable duplex (*Keene* et al. 1979). For a discussion of the possible sequence specificities involved in generating DI RNA stem sequences see Section 4.3.1 below.

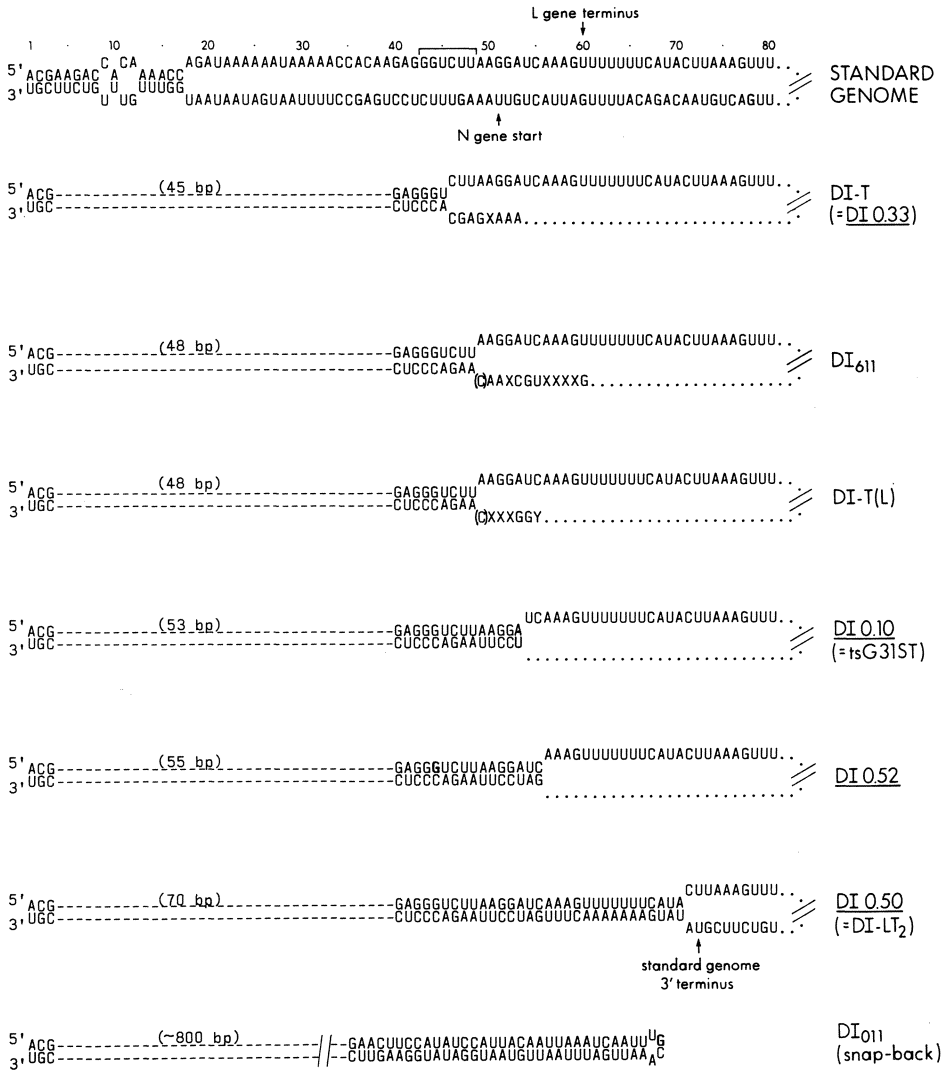

Fig. 2. Terminal sequences of VSV standard and DI RNAs. The dashed lines indicate complementarity of terminal DI RNA sequences, with the 5′-end sequence identical to that of the standard genome. The nucleotide sequence following the stem structure at the 3′-ends of the DI RNAs is not known beyond the few bases indicated (except for DI_{011} and *DI 0.50*). Residues in parentheses are tentative assignments whereas 'x' stands for undetermined. The putative promoter sequence at positions 43–48 from the 5′-end of the standard genome is indicated by a bracket. The sources of the various DI RNA sequences are as follows: DI-T, DI_{611}, and DI-T(L), *Schubert* et al. (1979); *DI 0.10, S. Nichol* and *J. Perrault*, unpublished; *DI 0.52, Hagen* and *Huang* (1981) and *D. Rao* and *A.S. Huang*, personal communication; *DI 0.50, Keene* et al. (1981a, b); DI_{011}, *Schubert* and *Lazzarini* (1981)

3.2.3 Snap-Back Hairpin Molecules

This kind of VSV DI RNA (Class I, types 2 and 3 in Fig. 1) was discovered independently in two laboratories by virtue of the odd behaviour of the molecules following denaturation protocols (*Lazzarini* et al. 1975; *Perrault* 1976). They appear to instantly snap-back into a double-stranded configuration after disruption of all hydrogen bonding by heat and/or denaturing solvents. If the molecules are first treated with ribonuclease to remove any putative single-stranded regions, they can then be denatured into separate plus and minus strands each equal to about one-half of the original mass of the molecule. Electron microscopic and nuclease studies further indicated that these molecules contain little single-stranded character and are most likely hairpin molecules obtained on deproteinization of a single-stranded ribonucleoprotein complex.

Further extension of these studies showed that snap-back hairpin type molecules of different sizes are relatively common in VSV DI isolates, and like most other VSV DI RNAs correspond to unique subsets of the L cistron (*Perrault* and *Leavitt* 1977a; *Schubert* et al. 1979; *Keene* et al. 1979). Analysis of the 5′- (*Johnson* and *Lazzarini* 1977a) and 3′-terminal residues (*Keene* et al. 1977) as well as more extensive hybridization and sequence analysis of the prototype DI_{011} snap-back RNA (*Schubert* et al. 1979) established that the molecules consist of a contiguous 5′-end segment of the L gene (minus strand) linked covalently at its own 3′-end to a plus strand complement of this same segment. Very recently, *Schubert* and *Lazzarini* (1981) determined the "turn-around" sequence which links the minus and plus strands of DI_{011} RNA (Fig. 2). This sequence bears no obvious relationship to other VSV DI RNA terminal sequences. It is, however, symmetrical such that breakage of a single phosphodiester bond between a G and C residue yields a perfectly base-paired duplex (estimated to be about 860 bp long).

Electron microscopic observations also indicated that ribonuclease-treated snap-back molecules (single-stranded region removed) are capable of circle and concatemer formation (*Perrault* and *Leavitt* 1977b). On this basis it was suggested that intact snap-back molecules contain inverted complementary sequences at both ends of the duplex. Hybridization studies and partial sequence determinations carried out in the author's laboratory have confirmed that this is indeed the case for DI tsG31 MT snap-back RNA but not so for DI_{C5ST} (*S.T. Nichol* and *J. Perrault* unpublished). The latter DI is most likely identical to DI_{011} of *Lazzarini* and colleagues since it was grown independently in the two laboratories from the same standard virus inoculum stock originating from a five times consecutively plaqued clone (*Holland* personal communication). There are therefore two distinct types of snap-back VSV DI RNAs (Fig. 1). The exact base sequence of the "turn-around" region in the tsG31 MT DI has not yet been determined. The ability of a small proportion of the ribonuclease treated DI_{C5ST} RNA duplex preparation to circularize or form large concatemers (*Perrault* and *Leavitt* 1977b) may have been due to contamination with other DI RNAs or to some unexplained property of these molecules. For a discussion of the possible origins of snap-back molecules, see Sec. 4.3.1 and 4.3.3.

3.2.4 Internal Genome Deletions

As mentioned previously the majority of VSV DI RNAs contain sequences contiguous with the 5′-end of the standard genome except for a short stretch at their 3′-ends. Early studies, however, showed one important exception. The HR (heat resistant) VSV Indiana strain gave rise to at least one LT (long truncated particle) DI preparation which maps at the 3′-end of the standard genome (see *Reichmann* and *Schnitzlein* 1979).

Furthermore, this HR-LT isolate is unusual in that it interferes heterotypically with the standard VSV New Jersey virus (see Section 5.4.1 below).

In addition, this HR-LT DI transcribes the four VSV mRNAs proximal to the 3′-end of the standard genome both in vitro (*Colonno* et al. 1977) and in vivo (*Chow* et al. 1977; *Johnson* and *Lazzarini* 1977b, 1978). This strongly suggests that the template HR-LT RNA contains the exact 3′-end terminal sequence of the infectious parent virus up to and including the G gene. However, high resolution gel analysis of denatured RNA species (*Perrault* and *Semler* 1979), as well as electron microscopic analysis (*Epstein* et al. 1980), revealed the presence of at least two types of molecules in the HR-LT isolate. Hybridization studies with the separated RNAs (*Perrault* and *Semler* 1979), using end-labeling techniques and DI RNA stems (corresponding to the 5′-end of the standard genome) led to the following conclusions: 1. the larger of the two HR-LT RNA species examined, denoted VSI HR ATCC *DI 0.50*, contains stems, approximately 65 bp long, which are derived from the 5′-end of the standard genome and its complement, as in other VSV DI RNAs previously examined; 2. the smaller (by about 10%) of the two HR-LT RNA species, denoted VSI HR ATCC *DI 0.46*, does not contain stem structures and its ends are identical to the original 5′- and 3′-end sequences of the standard genome, thus corresponding to an internal deletion; 3. the larger *DI 0.50* RNA also contains all or almost all of the sequences derived from the 3′-end of the standard genome present in the smaller *DI 0.46* RNA, and therefore also contains an internal genome deletion (see Fig. 1).

Epstein et al. (1980), using a different approach, reached many of the same conclusions regarding the two species of RNA in their preparations of the HR-LT DI. Only the larger species, denoted in their study DI-LT_2 (probably identical to *DI 0.50*), is capable of circularization suggesting stem structures. Sequencing techniques in addition revealed that both forms retain the 5′-end of the genome, but only the shorter linear species, denoted DI-LT_1 (probably identical to *DI 0.46*), retains the parental 3′-terminal sequence. A third study by *Clerx-Van Haaster* et al. (1980) also strongly suggests the presence of internal deletions in their HR-LT isolate, which was not characterized in terms of the number of RNA species present, but clearly shows conservation of T1 oligonucleotides from both the 3′- and 5′-ends of the standard genome.

In addition to the structural features described above, *Perrault* and *Semler* (1979) suggested, on the basis of hybridization studies, that *DI 0.50* RNA might contain the exact 3′-end sequence of the standard genome a short distance inward from its own 3′-end. This important feature was recently confirmed and extended by *Keene* et al. (1981a, b), who directly sequenced the 3′-end of their DI-LT_2 RNA and showed it to be an exact complement of the 5′-end of the standard genome for 70 nucleotides, followed by one noncoded A residue, and then immediately followed by the standard genome 3′-end sequence. The implications of these findings regarding polymerase activities in DI and standard VSV are discussed in Section 5.2 below.

Size estimates of the 5′-end standard genome segment conserved in the different HR-LT isolates from different laboratories vary considerably. *Perrault* and *Semler* (1979) estimated approximately 200 nucleotides for *DI 0.46* RNA, and approximately 500 nucleotides for *DI 0.50* RNA. *Epstein* et al. (1980) reported 320–350 nucleotides for their DI-LT_1, and possibly about 860 nucleotides for DI-LT_2 (the revised length estimate of the DI_{011} hybridization probe). *Chanda* et al. (1980) suggested approximately 450 nucleotides on the basis of duplexes generated by nuclease digestion of a full-length RNA transcript of their HR-LT isolate [denoted VSI HR ATCC *DI 0.67*(3′) in their study] annealed to the

standard genome RNA. It is not clear at the present time whether these differences reflect imprecision in the methods employed, or possibly variable size RNAs in the isolates of HR-LT DI. It should be noted here that all HR-LT stocks analyzed in the above studies were originally derived from the same isolate (*Petric* and *Prevec* 1970). The passage history in different laboratories, however, may have enriched for one or more given RNA species or generated additional genomic rearrangements. As discussed below (see Section 5.4.1) it is already clear that some DI can preferentially replicate as compared to others.

3.3 Other Negative-Strand Viruses

3.3.1 Sendai

The standard genome of the paramyxovirus Sendai is about 15 kb long and serves as template for the synthesis of six monocistronic mRNAs in vivo or in vitro (*Choppin* and *Compans* 1975; *Kingsbury* 1977). Its replication strategy is very similar to that of VSV. The earliest characterization of Sendai DI RNAs showed that they are smaller than standard virion RNA (sedimenting at 19S and 25S in contrast to 50S for standard RNA), are negative in polarity, and behave similarly to previously characterized VSV DI RNAs (*Kingsbury* et al. 1970; *Kingsbury* and *Portner* 1970). No further work on the properties of Sendai DI RNAs was published until several years later when they gained major importance in being the first reported to form circular molecules (*Kolakofsky* 1976).

A series of reports from *Kolakofsky* and colleagues have since provided several details of Sendai DI RNA structure, many of which shortly preceded and/or paralleled the developments in the similarly organized VSV system. In particular, Sendai DI RNAs were shown to contain stem structures approximately, 110–150 bp in length, which can be isolated by ribonuclease digestion (*Leppert* et al. 1977). As in the case of VSV (see Section 3.2.2 above) the 3′-end sequence of the DI RNAs is not found in the standard genome but represents a complementary copy of the 5′-end sequence common to both standard and DI particle RNAs (*Leppert* et al. 1977). Furthermore, the sequences of at least two DI particles RNAs, approximately 1200 and 2600 bases long, were shown to be contiguous with sequences at the 5′-end of the standard genome (except for the small stretch at the 3′-end represented in the stem structure). The length of independently generated Sendai DI RNAs was also examined under denaturing conditions and found to vary from about 670 to 7100 nucleotides (*Kolakofsky* 1979). So far, the genomic rearrangements observed in Sendai RNAs are analogous to those seen in VSV.

3.3.2 Influenza

Although the earliest biological studies on the generation of DI were carried out with influenza virus (*von Magnus* 1954) much less is known about their molecular biology than in the VSV system. This was in part due to the lack of a system for growing large amounts of influenza DI and difficulties in separating them from infectious virus (see review by *Nayak* 1980). More recent studies have employed cell lines which yield large amounts of virus and which can be infected under controlled conditions producing either standard virus only, or highly enriched DI preparations (*Lenard* and *Compans* 1975; *Nayak* et al.

1978; *Janda* et al. 1979; *Nakajima* et al. 1979). In addition, a direct biological assay system based on infectious center reduction was recently developed for quantitating influenza DI (*Janda* et al. 1979).

Early reports indicated that influenza DI contain reduced amounts of the larger viral RNAs (*Duesberg* 1968; *Choppin* and *Pons* 1970; *Nayak* 1972). However, further characterization of these RNAs did not progress until recent years when it was shown unambiguously that influenza virus is composed of eight RNA gene segments (*Palese* 1977; *Scholtissek* 1978). Three laboratories then reported the presence of extra small RNAs specific for influenza DI preparations (*Nayak* et al. 1978; *Crumpton* et al. 1978; *Nakajima* et al. 1979). Further studies showed that standard virus free from small RNAs can be isolated, and that such standard virus clones consistently generate DI-containing small RNAs on further serial passage (*Nayak* et al. 1978; *Janda* et al. 1979; *Ueda* et al. 1980).

The genomic content of single DI particles in influenza is not known. The loss of plaque-forming ability in undiluted passages of the virus did not correlate well with the loss of large RNA segments in at least one study (*Bean* and *Simpson* 1976). *Nayak* et al. (1978) attributed the decrease of infectivity to the nonspecific loss of one or more of the four largest viral RNA segments. Additional studies showed variability in reduction of these fragments in DI generated from different standard virus clones (*Janda* et al. 1979). Recently, *Pons* (1980) reported a good correlation between loss of segment 1 and loss of plaque-forming ability. *Crumpton* et al. (1981), on the other hand, find no such simple relationship. Further studies are needed to clarify these differences. Interfering ability, however, is directly correlated with the presence of virus ribonucleoproteins containing small RNAs (*Janda* and *Nayak* 1979), and shows small UV target size compared to infectivity (*Nayak* et al. 1978).

The replication strategy of influenza differs significantly from that of VSV and paramyxoviruses. In contrast to the latter, influenza does not code for enzymes capable of directly capping its mRNAs. Instead, the virions contain a nuclease (most likely virus coded) which cleaves small cap-containing fragments (10–14 nucleotides long) from the 5′-ends of host mRNAs in vivo (or added to in vitro reactions with purified virus) and uses these as primers to initiate transcription at the 3′-ends of the gene segments. This dependence on capped host mRNAs undoubtedly accounts for the requirement of a functional host RNA polymerase II for influenza virus replication (for a recent review of influenza transcription priming see *Krug* et al. 1981). Transcription of each gene segment also terminates about 20–30 residues from the 5′-end of the templates followed by polyadenylation. Thus, as in VSV, some yet uncharacterized regulatory switch must occur during virus growth to allow synthesis of full-size negative-strand templates. In addition, influenza is so far the only true RNA virus which produces spliced mRNAs, most likely using the host nuclear enzymes (*Lamb* and *Lai* 1980).

The influenza DI RNAs so far examined are negative-strand molecules (*Davis* and *Nayak* 1979). Reported sizes vary from as little as approximately 100 nucleotides for the smallest avian fowl plague virus RNAs (*Crumpton* et al. 1978) to molecules possibly as large as about 1200 nucleotides (slightly larger than virion segment 7) for the WSN strain of influenza (*Janda* et al. 1979). The sequence relationship between DI RNAs and individual virion RNA segments was studied by oligonucleotide fingerprinting (*Nakajima* et al. 1979; *Davis* and *Nayak* 1979; *Ueda* et al. 1980) and direct RNA sequencing (*Davis* et al. 1980). In all cases studied, the small RNAs are derived from one or the other of the three large polymerase genes (P_1, P_2 or P_3). In some instances, one or more extra

oligonucleotides not present in the virion segment of origin are observed. The most extensive study (*Davis* and *Nayak* 1979) showed that oligonucleotides from different size DI RNAs, originating from the same virion segment, are either completely overlapping or only partially overlapping. The latter observation led to the suggestion that some of these DI RNAs might correspond to internal deletions within genome segments. *Ueda* et al. (1980) also reported that two small RNAs originating from the same P_1 gene share none of the large unique oligonucleotide spots from this segment. Paradoxically, only one of these RNAs is associated with a reduction of the P_1 gene segment while the other shows a reduction of the P_2 gene. Only one report (*Crumpton* et al. 1979) has claimed that influenza DI RNAs might also originate from segments other than the P genes, but the interpretation of the oligonucleotide maps is clouded by the fact that the analysis was carried out with mixtures of RNA segments.

Sequence determination of the first 13 bases of the 5′-end and the first 12 bases at the 3′-end of the P genes (these sequences are essentially identical in all virion segments) and three DI RNAs (derived from P_1) show complete homology (*Davis* et al. 1980). These results clearly establish the presence of internal genome deletions in at least these three DI RNAs which are all derived from the same standard virus clone. Whether this is the case for most or all influenza DI RNAs remains to be established. Very recently, *Nayak* and colleagues (personal communication) determined the complete sequence of a cloned double-stranded DNA copy derived from the smallest of these three influenza DI RNAs (409 bp long). The bases flanking the site of internal deletion (very near the middle of the molecule) bear no homology to the consensus sequence for a splice site in eucaryotic mRNAs including the splice junction of the NS_2 mRNA derived from virion segment 8 of influenza virus (*Lamb* and *Lai* 1980). The implications of these recent findings are discussed in Section 4.3.2.

3.4 Positive-Strand Viruses

3.4.1 Togaviruses

All of the information available on the standard genome sequences represented in togavirus DI originates from studies of two alphaviruses, Sindbis (SV) and Semliki Forest virus (SFV) (for detailed review on all aspects of togavirus DI, see *Stollar* 1979 and 1980). The standard genome of these viruses (about 13 kb long) is capped at the 5′-end, polyadenylated at the 3′-end, and contains one active site near its 5′-end for the translation of a non-structural protein precursor. In addition, a subgenomic 26S RNA (about 4.2 kb long), which contains the translation initiation site for a structural protein precursor, is synthesized in infected cells. This subgenomic RNA corresponds to a contiguous segment at the 3′-end of the genome and its synthesis most likely results from internal initiation on a complementary negative-strand RNA copy of the full-size genome (*Dubin* et al. 1979; *Pettersson* et al. 1980). The latter also serves as a template for the synthesis of progeny RNA (see reviews by *Kääriäinen* and *Soderlund* 1978, and *Kennedy* 1980). The complete nucleotide sequence of the 26S mRNA of both SFV and SV was recently determined (*Garoff* et al. 1980a, b; *Rice* and *Strauss* 1981; *Ou* et al. 1981). The sequence results show unambiguously that the genome length RNA and the subgenomic 26S RNA share identical sequences extending to the 3′-terminus (*Ou* et al. 1981). The mechanism of gene

expression in this virus group is seemingly more complex than that seen in rhabdo- or paramyxoviruses. This feature is perhaps responsible for the greater complexity of structures seen in togavirus DI RNAs.

The first reports on the nature of togavirus DI RNAs described the appearance of novel RNA species in cells infected with high passage SV (*Shenk* and *Stollar* 1972; *Weiss* and *Schlesinger* 1973; *Eaton* and *Faulkner* 1973). DI RNAs of SFV were first analyzed by *Bruton* and *Kennedy* (1976). Several different size species of DI RNAs are observed in both SV and SFV grown in vertebrate or invertebrate cell lines. These range from about 1.7 kb to about 10 kb (*Johnston* et al. 1975; *Kowal* and *Stollar* 1980). Other published values for both SV and SFV DI RNAs fall between these extremes (see *Stollar* 1979). Interestingly, several laboratories reported that larger DI RNAs decrease progressively in size as serial undiluted passage of virus stocks is continued (*Johnston* et al. 1975; *Guild* et al. 1977; *Stark* and *Kennedy* 1978). The implication of these findings in relation to interference with standard virus replication is discussed below (see Section 5.5).

The polarity of SV and SFV DI RNAs was determined by hybridization experiments with various combinations of particle RNAs and single- and double-stranded RNAs isolated from infected cells (*Weiss* et al. 1974; *Bruton* et al. 1976; *Guild* et al. 1977), as well as oligonucleotide mapping (*Kennedy* 1976; *Kennedy* et al. 1976; *Dohner* et al. 1979). These studies showed that the DI RNAs are polyadenylated, positive strands, and contain virus-coded sequences only.

The hybridization and oligonucleotide studies also served to establish maps of the genome sequences represented in the various DI RNAs. A common theme in all of these is conservation of both 5′- and 3′-end sequences of the standard genome, although in some cases, one of the conserved ends appears to be very small in size (*Weiss* et al. 1974; *Kennedy* et al. 1976). Based on comparison of unique oligonucleotides in DI RNAs vs standard RNA, and the relative map position of these oligonucleotides, the sequence arrangement shown in Figure 3a was proposed for SFV DI RNAs (*Kennedy* 1976; *Stark* and *Kennedy* 1978). On the basis of RNA-RNA hybridization studies, and assuming a simple internal deletion, *Guild* and *Stollar* (1977) proposed the arrangement shown in Fig. 3b for SV DI RNAs.

The studies discussed so far indicate a somewhat larger representation of standard genome sequences derived from the 5′-end in various togavirus DI RNAs. In contrast to this, a SV DI RNA, about one-fifth the size of the genome, was shown to contain sequences originating mostly from the 3′-end of the standard genome, with only a short 5′-end sequence of undetermined size (*Weiss* et al. 1974; *Kennedy* et al. 1976).

It seems clear from the above studies that many togavirus DI RNAs represent internal deletions of the standard genome. However, some published reports suggested more complex situations and recent analysis of one particular SFV DI shows this to be the case. On the basis of oligonucleotide maps, both *Stark* and *Kennedy* (1978) and *Dohner* et al. (1979) suggested the presence of two or more noncontiguous internal deletions in at least one DI RNA from SFV and SV respectively (see Fig. 3a). Furthermore, although the report of *Kennedy* (1976) indicated molar yields of oligonucleotides from SFV DI RNAs, *Dohner* et al. (1979) reported wide variations in these yields for the one SV DI RNA examined.

Pettersson and colleagues (personal communication) have now determined the detailed sequence organization of one SFV DI RNA as deduced from a molecular cloned double-stranded DNA copy (about 1650 bp long). The results obtained show

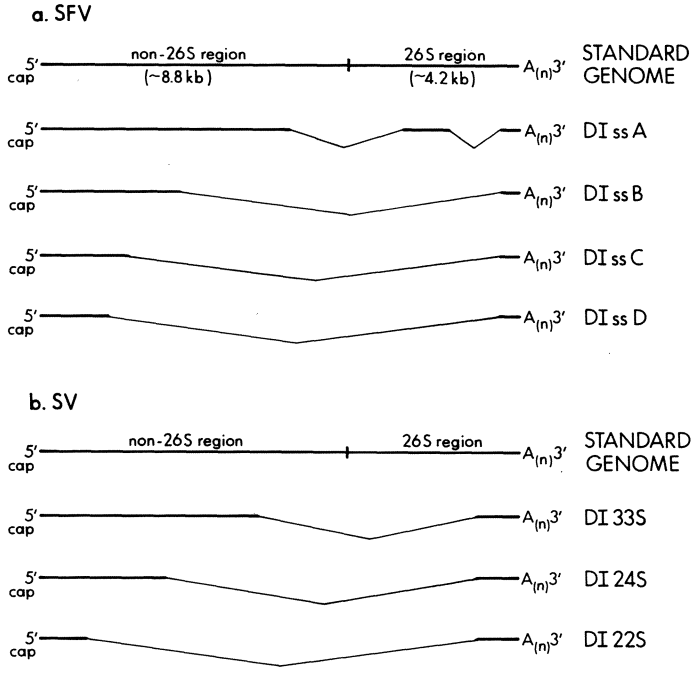

Fig. 3. Proposed sequence organization of alphavirus DI RNAs. The solid lines represent conserved regions of the genome whereas light lines indicate deleted portions. Evidence for more complex sequence rearrangements is discussed in the text

three repeat sequences 484 nucleotides long, with a small insert sequence between two of these, as well as within one of the repeats. All the sequences of this clone appear to be derived from within the 5′-two-thirds of the standard genome (non-26S RNA sequences) except for a stretch of 106 identical residues at the 3′-ends of both the standard and DI RNA. The cloned DNA copy was obtained from a heterogeneous mixture of DI RNAs sedimenting at about 18S in a sucrose gradient. S1 nuclease mapping experiments, however, showed that this cloned DNA contains a faithful and uninterrupted complementary copy of at least one SFV DI RNA in this mixture. The preparation of DI RNAs was obtained following the tenth high multiplicity passage of a standard SFV stock which already showed the presence of DI RNAs at passage four. The cap-containing T1 oligonucleotides of this "18S" RNA mixture were also found to be heterogeneous with the general sequence cap-AU$(AU)_n$CAUG, where n = 4 to 8 (*Pettersson* 1981). This cap sequence is different from that reported for both standard and 26S SFV RNAs, i.e., cap-AUG and cap-AUUG respectively (*Wengler* et al. 1979; *Pettersson* et al. 1980). Therefore, the exact 5′-end terminal sequence of the standard genome is apparently not conserved in some of these DI molecules (the heterogeneity of the DI 5′-cap sequences, however, could conceivably be generated by a nontemplated mechanism). The cap sequences of two SV DI RNAs were recently reported to resemble, at least in part, the cap sequences of standard virus in one case and that of 26S subgenomic RNA in the other (*Kowal* et al. 1980). The above results clearly show that extensive genome rearrangements, including

deletions, duplications, insertions and possibly others, can occur during the genesis of togavirus DI RNAs. Future experiments should clarify whether the heterogeneity and more complicated rearrangements of these DI RNAs evolve from simpler forms during multiple passages of virus stocks (see discussion Section 4.3.2).

3.4.2 Picornaviruses

Only two picornaviruses, poliovirus, and mengovirus were reported to generate DI (*Cole* et al. 1971; *McClure* et al. 1980). The relative difficulty in generating such particles (by long term passage at high multiplicities of infection) is unusual in comparison to other RNA viruses. The reasons for this are unclear but may be related to relatively low levels of interference and to the choice of host cell which appears to influence the production of picornavirus DI (*McClure* et al. 1980; see also Section 4.1). Poliovirus live vaccines also do not generally contain DI (*McClure* et al. 1980) in contrast to previous suggestions (*McClaren* and *Holland* 1974).

Almost all of the molecular studies of picornavirus DI were carried out with the type I Mahoney poliovirus strain originating from the same laboratory (*Cole* et al. 1971; *Nomoto* et al. 1979; *Lundquist* et al. 1979). The standard genome of poliovirus (approximately 7500 nucleotides long) is covalently-linked to a small virus-coded protein, VPg, at its 5′-end and is polyadenylated at its 3′-end. It contains a major initiation site for synthesis of a large polyprotein precursor (~ 200,000 daltons mol. wt.) near its 5′-end, and possibly a second initiation site of unknown location and coding potential (*Celma* and *Ehrenfeld* 1975). The standard genome is the only mRNA so far detected intracellularly and is replicated via synthesis of a full-size complementary RNA copy (for a review of picornavirus gene expression and replication see *Agol* 1980 and *Kitamura* et al. 1980). The complete sequence of the type I Mahoney poliovirus genome has recently been determined (*E. Wimmer,* personal communication).

The studies of *Cole* and colleagues (*Cole* et al. 1971; *Cole* and *Baltimore* 1973a, b, c) on the nature of poliovirus DI RNA led to the following conclusions: 1. the three independent isolates of DI particles studied all contain RNAs differing slightly from each other in size and are about 15% smaller than the standard genome; 2. about 87% of the standard genome sequences are conserved in the DI RNAs isolated, as determined by competition hybridization experiments with the replicative form of the standard genome; 3. deleted portions of the standard genome correspond to sequences coding for capsid polypeptides (mapping towards the 5′-end of the genome), since cells infected with DI particles in the absence of standard virus give rise to translation products missing only about one-third of the capsid protein precursor. The latter observation was confirmed and extended in subsequent studies (*Humphries* et al. 1979; *Lundquist* et al. 1979; *Phillips* et al. 1980). The findings of *Villa-Komaroff* et al. (1975) also suggested that poliovirus DI RNAs correspond to internal deletion since the DI RNA-directed polyprotein precursor synthesized in vitro contains the same initiation tryptic peptide as found for standard virus RNA. Furthermore, *Humphries* et al. (1979) showed that DI RNA contains the additional in vitro protein initiation site discovered earlier for the standard genome. The latter investigators also demonstrated that DI RNA can serve as template for the synthesis of VPg in vivo.

Further studies on the map location of internal deletions in poliovirus DI RNAs were carried out using a variety of methods. *Nomoto* et al. (1979) employed three methods: 1. limited digestion with ribonuclease III to produce large fragments of standard and DI

RNAs (map order deduced from the presence or absence of poly A); 2. comparison of oligonucleotide fingerprints; and 3. electron microscopy of RNA-RNA heteroduplex molecules formed by annealing DI RNA and standard genome replicative form molecules. These studies confirmed that the deletions in all three DI RNA preparations originally isolated by Cole and colleagues are in the 5′-terminal half of the standard genome, and are all missing the same four unique oligonucleotides. In addition, the report showed that the RNA molecules in one of the DI preparations are about 12% shorter than the genome but are somewhat heterogeneous since the location and extent of individual deletions in heteroduplex molecules varied (located approximately 1300 to 3100 bases from the 5′-end). A more detailed electron microscopic study of heteroduplex molecules from poliovirus DI RNAs generated from the same poliovirus strain, and possibly representing independent isolates (the standard virus, however, was not cloned) provided further evidence for heterogeneity (*Lundquist* et al. 1979). The deletions in this case were shown to be about 16% of the standard genome but were found to be located anywhere from about 500 to about 3800 bases from the 5′-end. Some of the deletions examined are not contiguous and represent double and even triple deletions (still adding to the same 16% or so overall deletion). It was also noted that the DI RNA population is not static on further passage but differs in the relative amounts of individual species suggesting a basis for the extensive heterogeneity observed.

Although several laboratories experienced great difficulty in generating DI in picornaviruses, other than the poliovirus strain mentioned in the studies above, the results of *McClure* et al. (1980) suggested that this problem may in part result from the small size of the deletions generated in most cases. The deletions detected in two different clinical isolates of poliovirus and a laboratory stock of mengovirus are reproducibly in the range of 4–6%, whereas even stocks cloned twice consecutively from the poliovirus strain employed in all previous studies, repeatedly give rise to the larger deletions. As pointed out by a number of authors (*Cole* 1975; *Lundquist* et al. 1979; *McClure* et al. 1980) it is likely that packaging restraints govern which size molecules are eventually encapsidated in picornavirus DI and this may explain the differences in deletion sizes for the different virus strains (see Section 5.3). The map locations of the small picornavirus deletions have not yet been determined. The poly(C) tract of mengovirus (approximately 100 residues in length), located near the 5′-end of the genome, is conserved in its DI RNA (*McClure* et al. 1980).

The marked population heterogeneity of picornaviruses DI RNAs has somewhat complicated a more detailed analysis of the recombination sites in these molecules. Molecular cloning of DNA copies from these genomes will undoubtedly be reported soon and rapid progress is likely to follow.

3.5 Double-Strand Viruses

Reovirus was the first double-strand virus reported to generate DI (*Nonoyama* et al. 1970). Members of the reovirus group contain ten double-strand RNA segments each coding for a unique viral gene protein. Each segment is completely transcribed by the virion-associated transcriptase into a capped plus-strand mRNA which in turn serves as template for synthesis of the double-stranded progeny RNA (*Joklik* 1980). *Nonoyama* and *Graham* (1970) reported that five independent clones of type 3 reovirus each give rise to

DI lacking the largest virion segment now known to code for the λ_3 polymerase component. *Schuerch* et al. (1974) using ts mutants of type 3 reovirus reported that one of these generates DI with virion segment 3 missing, whereas another generates DI with both segments 1 and 3 missing. The same two virion segments were again implicated in DI recovered from infected animals (*Spandidos* and *Graham* 1976) and persistently infected cell cultures (*Ahmed* and *Graham* 1977). Aside from some transcriptional aspects (see Section 5.2) no further detailed molecular studies have yet been carried out with these various DI isolates. In particular, efforts should be made to look for small RNA segments which might represent internal deletions within gene segments analogous to influenza virus DI. Two such possible internally deleted segments have recently been observed by *Ahmed* and *Fields* (1981). The "light defective" particles obtained from persistently infected cells might also contain such RNAs (*Ahmed* and *Graham* 1977).

Virus-like particles in fungi are considered to be bona fide viruses although they lack the criterion of infectivity (see review by *Bruenn* 1980). The well-characterized yeast double-stranded *Saccharomyces cerevisiae* virus (ScV) contains two genome segments, L and M, separately encapsidated. The virus was shown to give rise to smaller RNAs in suppressive yeast strains (*Vodkin* et al. 1974; *Tzen* et al. 1974). Since the smaller RNAs appear to replace the M segment whenever present in the same cytoplasm, they are considered analogous to DI (*Bruenn* and *Kane* 1978; *Fried* and *Fink* 1978). The L genome fragment, about 4500 bp long, codes for the major capsid protein and the M segment, about 1800 bp long, codes for the "killer toxin" (*Bostian* et al. 1980). For a detailed discussion of the relationship between sensitivity to toxin, synthesis of toxin, and segregation of suppressive sensitive strains, the reader is referred to *Bruenn* (1980).

The size of the various ScV DI RNAs observed ranges from about 540 to 1500 bp. All of these are derived from M, as demonstrated by comparative oligonucleotide fingerprint analysis (*Bruenn* and *Kane* 1978; *Kane* et al. 1979), by electron microscopic heteroduplex analysis (*Fried* and *Fink* 1978), and by 3′-end sequence analysis (*Bruenn* and *Brennan* 1980). All of the DI RNAs examined conserve sequences from both the 5′-and 3′-ends of the M RNA segment and are thus internal genome deletions. Some of these appear to regularly give rise to tandemly duplicated molecules. The sequences flanking the deletion sites have not yet been determined.

Although relatively little is known regarding the replication strategy of ScV (it is probably somewhat similar to reovirus) this system is attractive for DI studies because well defined gene mutations, some of which map in the host nucleus, specifically affect the replication of the M virus segment. In addition, this system represents an extreme case of virus persistence since transmission occurs only during mating. Other less well studied fungal virus systems also share properties similar to ScV and most likely produce similar DI RNAs (*Bruenn* 1980).

4 Origin of DI RNAs

4.1 Host Cell Effects on the Generation of DI RNAs

The passage number at which virus yields become greatly diminished, as a result of DI production, not only varies widely for different viruses, but also for the same virus grown in different host cells. This general phenomenon could result a priori from a host effect

on the de novo generation of DI from standard virus and/or from modulation of the amplification properties of DI. Many published studies on this subject are somewhat confusing because they have not attempted to differentiate between these two alternatives. Effects on DI generation (this term will be used throughout this article to denote the events which give rise de novo to a DI RNA molecule, either from a standard virus genome or from a previously existing DI molecule) are understandably much more difficult to measure.

Obviously, many different types of molecules with particular genome sequence rearrangements could be generated at low frequencies during virus replication. The ones that concern us, however, are only those which are capable of amplification, i.e., replication and enrichment. Claims that DI generation per se is specifically altered by the host must obviously eliminate explanations involving replication and/or interference effects. The latter two processes are clearly affected by the host cell and are discussed in Section 5.1.

The only DI system in which some attempt was made to measure the de novo rate of DI generation is that of VSV. *Holland* et al. (1976b) estimated this to be about 10^{-7} to 10^{-8} per infectious particle replication in baby hamster kidney (BHK_{21}) cells. This estimate was made possible because of the apparent random nature of individual DI particle generation. Whenever a given VSV plaque clone is grown to a seed stock containing more than about 10^7 to 10^8 plaque forming units (pfu) per ml, it invariably gives rise to the same array of DI species even if independent high multiplicity passage series are carried out. However, if the initial virus plaque (containing about 10^6 pfu or less) is not grown to a seed stock first, but used directly to initiate independent series of undiluted passages, a different array of DI RNA species appears in each case (*Holland* et al. 1976a). This crucial observation shows that, in addition to the rather random character of DI generation, many of the VSV DI RNA molecules amplified, whether large or small, possess similar interfering and replicating properties.

Others have reported that single VSV clones have a genetic capacity to regularly generate the same DI species in a given cell type (*Reichmann* et al. 1971; *Kang* et al. 1978a). However, these studies were carried out with VSV pools already contaminated with seed quantities of DI and the results therefore do not support a viral genetic basis for the type of VSV DI generated (*Holland* et al. 1980). A similar randomness in the type of DI generated from clonal virus pools is also observed with influenza DI (*Janda* et al. 1979) and Sendai DI (*Kolakofsky* 1979).

A possible host effect on the frequency of VSV DI generation was also noted by *Holland* et al. (1976a) who showed that a particular HeLa cell line fails to produce DI after 27 serial undiluted passages and does not replicate added DI generated in BHK_{21} cells. Only very few DI were detected in the HeLa lysates by subsequent amplification in BHK_{21} cells. This effect, however, is also consistent with a low amplification rate.

Stark and *Kennedy* (1978) likewise reported lack of SFV DI production in the same HeLa cell line after more than 200 serial passages. More recently, *McClure* et al. (1980) showed lack of mengovirus DI production specific for this same host cell. SFV DI or mengovirus DI grown in other hosts, however, were reported to replicate well in this HeLa host. These results are therefore suggestive of specific effects at the generation level. Curiously, *Kang* and *Tischfield* (1978) also claimed that a human cell line is capable of suppressing the production of VSV DI in eight karyotypically different human-mouse hybrid cell lines, without influencing the replication of DI produced in other host cells.

This is not, however, a general property of human cell lines, since poliovirus DI can be generated in the HeLa cell line mentioned above (*McClure* et al. 1980), and SFV DI can also be generated in a different strain of HeLa cells (*Stark* and *Kennedy* 1978). Moreover, HeLa cells can produce VSV DI at times but unpredictably so (*Holland* et al. 1978; *J. Perrault* and *P. Schnarr*, unpublished).

Igarashi and *Stollar* (1976) were unable to find evidence for the production of SV DI during serial undiluted passage in *Aedes albopictus* cells. Later work showed, however, that alphavirus DI are produced in persistently infected mosquito cells (*Eaton* 1977). They also accumulate on serial passage if a longer incubation time is used (*King* et al. 1979) or if individual clonal cell populations are first selected for high virus yield (*Logan* 1979; *Stollar* 1979; *Holland* et al. 1980; *Tooker* and *Kennedy* 1981). VSV DI are also generated in high yields from mosquito cells (*Gillies* and *Stollar* 1980a). It thus seems probable that arboviruses in general are also capable of generating DI in invertebrate hosts.

A more striking and direct role for the host cell in the generation of VSV DI was proposed by *Kang* and *Allen* (1978) and *Kang* et al. (1978b). These authors claimed that pretreatment of a variety of vertebrate host cells for 24 h with actinomycin D completely abolishes the generation of VSV DI even after 12 undiluted passages, whereas control cells show a large accumulation of DI by two or three passages. No interference activity could be detected even though, as shown previously and confirmed in this study, actinomycin D has no effect on VSV standard or DI particle replication. These data clearly suggest that a cellular factor, requiring renewed transcription and possibly translation within 24 h, is essential for the generation of VSV DI. Unfortunately, these important claims have not been confirmed despite serious effort by a number of other laboratories (*Youngner* et al. 1981; *Reichmann,* personal communication; *Horodyski* and *Holland,* personal communication; *J. Perrault* and *P. Schnarr*, unpublished).

The various rates of DI accumulation in different host cells observed for VSV (*Huang* and *Baltimore* 1970; *Coward* et al. 1971; *Potter* and *Stewart* 1976; *Kang* et al. 1978a; *Youngner* et al. 1981), paramyxoviruses (*Kingsbury* and *Portner* 1970; *Roman* and *Simon* 1976), influenza (*Choppin* 1969; *De and Nayak* 1980), and togaviruses (*Darnell* and *Koprowski* 1974; *Stark* and *Kennedy* 1978) are difficult to assess in terms of DI generation versus amplification because all of the above studies were initiated with virus stocks already contaminated with seed quantities of DI. Furthermore, replication rates and interference levels were not measured independently of generation. Most or all the above host effects could thus be explained on the basis of a cellular influence on the amplication properties of DI RNAs (see Section 5.1).

An interesting observation analogous to a host effect on DI production was also reported by *Winship* and *Thacore* (1979). Undiluted passage of VSV in African green monkey kidney cells results in DI production but the latter are not present if the cells are first infected by Shope fibroma virus (ShFV) in the presence of hydroxyurea which inhibits the poxvirus replication without affecting VSV synthesis. Yields of infectious VSV are similar under both conditions suggesting a specific effect on DI production. As in previous cases, however, the results can most likely be explained by effects on DI replication and/or interference. Nevertheless, this system offers a promising approach in attempting to identify a putative host cell factor(s) specifically affecting VSV DI RNA replication.

A possible host cell effect on the kind of SFV DI RNA produced, rather than the general frequency at which DI are generated, was also reported by *Stark* and *Kennedy*

(1978). As mentioned previously (Section 3.4.1) larger togavirus DI RNAs appear to decrease progressively in size on continued passage. In the case of SFV, the decrease in size is consistent with a sequential series of deletions leading to subsets of smaller DI RNAs up to a "limit size" DI. In the pig kidney cell line (PK-15), the smallest DI RNA detected does not undergo the two additional deletions observed regularly in other host cells, even when passaged 32 times beyond the "limit passage". A possible interpretation is that this host cell lacks factors necessary for generating these deletions. Alternatively, the host cell could influence the selection of preexisting DI RNAs as observed in other systems (see Section 5.1).

Overall, the evidence presented above indicates that the host cell could sometimes influence the frequency and possibly the nature of DI generated de novo. However, it is impossible to rule out that all these effects occur at the level of DI amplification subsequent to their initial appearance. The weight of the evidence strongly argues that the nature of various DI molecules is more directly under virus than host cell control. The genetic make-up of an infectious virus might therefore be expected to influence directly the nature and/or rate of DI generation. Surprisingly, very few studies have addressed themselves specifically to this question. The claims regarding a standard virus clonal influence on the type of VSV DI produced and the probable alternative explanation have been discussed above already. *McClure* et al. (1980) on the other hand reported that only one of the four distinct isolates of poliovirus type 1 examined does not produce DI in HeLa cells. Furthermore, characteristic deletion sizes in DI RNAs from each of two isolates are reproducibly obtained in this same cell line even after repeated cloning to eliminate previously existing DI. These observations, which could be explained by several genetic differences between the isolates in terms of generation, could also be due to differences in stability of capsids containing the shorter RNAs. These studies further illustrate the difficulties encountered in trying to pinpoint the basis for differences in DI accumulation observed in different virus-host systems.

4.2 The Unlikely Provirus Hypothesis

Before discussing specific models for the generations of DI RNAs, it is important to consider first whether such recombination events could possibly occur at the DNA level. One laboratory in particular has published a number of reports claiming that several negative-strand RNA viruses, including measles, VSV, simian virus 5, LCM, and possibly influenza, as well as two positive-strand togaviruses, SV and tick-borne encephalitis, can integrate DNA copies of their genome into various host cells (*Zhdanov* and *Parvanovich* 1974; *Zhdanov* et al. 1974; *Zhdanov* 1975; *Gaidamovich* et al. 1978). The evidence for this claim is based mostly on DNA transfection experiments (giving rise to recoverable infectious virus) and detection of cellular DNA copies of the virus genome with labeled nucleic acid probes. A similar claim of DNA transfection using cells infected with the respiratory syncytial paramyxovirus (RSV) was also reported (*Simpson* and *Inuma* 1975). Three different mechanisms were suggested to account for these observations: 1. a gene product interaction between a negative- or positive-strand virus and a latent retrovirus; 2. a cellular reverse transcriptase-mediated integration event (*Zhdanov* 1975); or 3. a mutational or recombinational event (with a retrovirus genome or cellular genome) yielding an RNA virus variant with reverse transcriptase activity (*Furman* and *Hallum* 1973; *Simp-*

son and *Inuma* 1975). Although these ideas are intriguing possibilities, the weight of currently available evidence strongly argues against these claims.

This counterevidence includes DNA transfection attempts with cells persistently infected by VSV, RSV, SV, LCM, measles, mumps, influenza, and rabies (*Holland* et al. 1976c; *Igarashi* et al. 1977; *Pringle* et al. 1978) as well as attempts to detect proviruses by hybridization experiments with DNA from cells persistently infected with VSV, SV, measles, and rubella (*Holland* et al. 1976c; *Friedman* and *Costa* 1976; *Igarashi* et al. 1977; *Haase* et al. 1977; *Norval* 1979). The experiments of *Holland* et al. (1976c), in particular, are noteworthy in that the VSV carrier culture was well characterized, with essentially all cells remaining infected for over two years, and at the limit of detection contained less than one DNA provirus copy per 40 cells. Earlier claims of provirus DNA copies (detected by hybridization) in the yeast ScV system (*Shalitin* and *Fischer* 1975; *Vodkin* 1977) have also been refuted (*Wickner* and *Liebowitz* 1977; *Hastie* et al. 1978).

It is clear from the above studies that classic RNA viruses can associate with host cells for long periods of time without recourse to DNA integration. However, it is not possible to rule out that, under special and rare circumstances, an integration phenomenon can take place. It will be interesting to see whether biologically active proviral DNA copies of these RNA viruses can be constructed in the future by genetic manipulation in vitro. At the present time, we can safely assume that the deleted genomes in DI of RNA viruses are generated by events occurring at the RNA level, at least under most circumstances.

4.3 Purposeful Error-Prone Virus Polymerase

4.3.1 Terminal Sequence Complementarity and the Copy-Back Model

The very nature of DI RNAs suggests strongly that they originate from low frequency events during the replication or transcription of standard virus RNAs. Although we can view these events as "errors" in contrast to the dominant "fidelity" of the replication process, they are as purposeful as any other type of mutations in biological systems.

It is important to note here that trivial explanations for the generation of DI RNAs, such as RNA chain scission or premature termination during synthesis, are insufficient by themselves to explain the conservation of genomic ends or the presence of inverted terminal complementarity. Thus, fragmentation of VSV ribonucleoproteins by sonication, or chemical mutagenesis and UV irradiation of standard virus, does not lead to DI production (*Holland* et al. 1976a).

The unexpected features of terminal sequence complementarity in VSV and Sendai DI RNAs (see Section 3.2.2 for details) provide the strongest argument for the involvement of virus polymerases in the origin of DI RNAs. These observations led both *Kolakofsky* and colleagues (*Leppert* et al. 1977), and *Huang* (1977) to propose a "template-switching" and "copy-back" model for DI generation in negative-strand viruses (Fig. 4). The essential feature of this model is a viral replicase detaching from its template (while remaining attached to the nascent chain), and then binding back near the 5′-end of the nascent chain to initiate copying in the reverse direction. Since most VSV and all Sendai DI RNAs examined so far retain a large fragment from the 5′-end of the standard genome, it is assumed that such a copy-back event would occur during synthesis of minus strands.

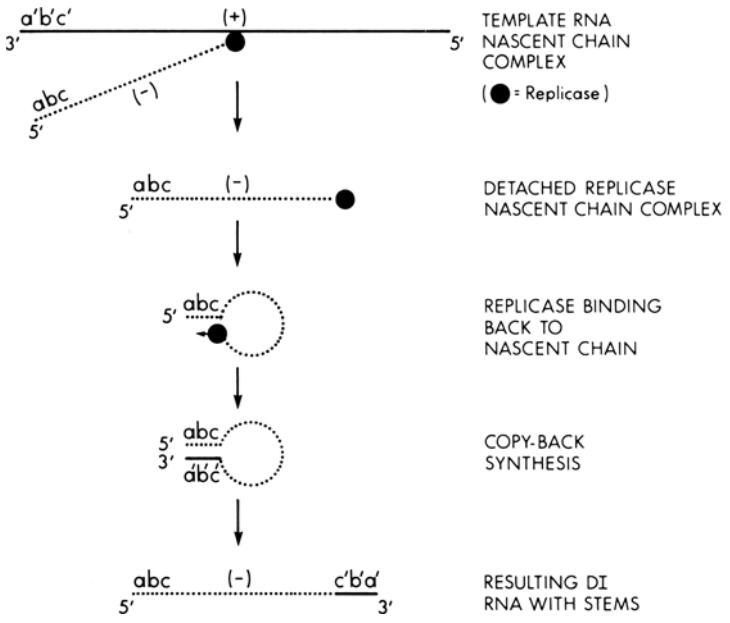

Fig. 4. Kolakofsky-Huang copy-back model for the generation of DI RNAs. The terminal sequences abc and their complement c′ b′ a′ in the resulting DI RNAs can anneal to each other (after isolation from the ribonucleoprotein template structure) to give rise to characteristic panhandles or stems

The Kolakofsky-Huang model raises some interesting questions regarding specificities of the "fall-off" sites on the template as well as the "resumption" sites of synthesis on the nascent chains. So far, only short stem structures (45–70 bp) or large hairpin snap-back molecules have been observed in VSV and Sendai DI RNAs (see Figs. 1 and 2). This suggests strongly that only sites close to the ends of nascent molecules are competent for resumption of copy-back synthesis. In contrast, judging from the extensive variability in size of the 5′-end standard genome segment conserved in various VSV and Sendai DI RNAs, the fall-off points may well be relatively random at least to a point about halfway down the genome. In this context, it should be remembered that replication of both plus and minus strands of rhabdo- and paramyxoviruses takes place on helical nucleocapsid templates. The tightly bound proteins of these templates may be displaced locally and transiently during RNA synthesis but "naked" RNA molecules are not detectable during RNA replication (*Soria* et al. 1974; *Hill* et al. 1979). Furthermore, the nascent chains are very rapidly assembled with newly synthesized core proteins. This protein association appears to completely protect the RNA from ribonuclease digestion (*Hill* et al. 1979) and extends to within at least 5 bases from both ends of Sendai DI particle nucleocapsids (*Lynch* and *Kolakofsky* 1978). Core protein-template RNA interactions may thus play a major role in replicase fall-off and resumption sites. The VSV and Sendai polymerases undoubtedly recognize some nucleotide sequence features but there would seem to be little chance for dependence on an extensively base-paired secondary structure in the

template. With this in mind, we can now examine possible nucleotide sequence specificities of the copy-back model.

Lazzarini and co-workers have provided a large share of the information available regarding sequences flanking the recombination sites in VSV DI RNAs. On the basis of their data on three independently isolated DI RNAs *Schubert* et al. (1979) proposed a specific RNA polymerase resumption site near the 5′-end of VSV genomic RNA. The sequence 5′-GGUCUU-3′, at positions 43 to 48 from the 5′-end of the standard genome (see Fig. 2) is also present at positions 4–9 at the 3′-ends of all DI RNAs (except *DI 0.46* = DI-LT_1) and at the 3′-end of standard genome plus strands. This hexamer sequence may thus be at least part of a putative promoter recognition site.

To account for the small differences in the precise location of the copy-back site (positions 45, 48, and 48 in the three DI RNAs examined in their study) *Schubert* et al. (1979) suggested two possibilities: 1. nucleotides copied just prior to replicase fall-off are, sometimes by chance, complementary to those adjacent to the polymerase recognition site; and 2. slippage of the RNA polymerase can occur in analogy to procaryotic promoter sites (see *Rosenberg* and *Court* 1979). In addition, a more general form of this model was proposed allowing inter- as well as intramolecular changes in template which could also explain internal deletions (see Section 4.3.2 and 4.3.3).

The copy-back model, with the added features of random fall-off and sequence specific resumption sites of synthesis, would seem to offer a coherent explanation for the origin of terminal sequence complementarity and internal deletions in DI RNAs. However, more recent work in the VSV system casts some doubt on the universal applicability of this model. Stem lengths in more recently characterized VSV DI RNAs (see Fig. 2) are somewhat longer (53, 55, and 70 bp) than the ones previously studied. The proposed resumption site for copy-back synthesis may therefore be less localized than anticipated. Furthermore, the sequence of the "turn-around" region in the snap-back DI_{011} (*Schubert* and *Lazzarini* 1981) which can be equated with the resumption site in this DI RNA, bears no homology at all to the putative VSV polymerase promoter (Fig. 2).

A quite different model was proposed by *Schubert* and *Lazzarini* (1981) to explain the origin of DI_{011}. The features of this model include the following: 1. replication complexes (synthesizing minus strands) contain more than one nascent chain; 2. individual replicase molecules move at variable speed along the template allowing a faster one to "tailgate" a slower one; 3. the trailing polymerase switches templates and starts synthesis (independent of the putative VSV promoter site) on the daughter strand attached to the leading polymerase; 4. this strand switching takes place exactly symmetrically at the replication fork. It should be noted here that the origin of the snap-back RNA structure proposed by *Perrault* and *Leavitt* (1977b) for type 3 molecules (Fig. 1) does not require any significant deviation from the original copy-back model. As pointed out by *Leppert* et al. (1977), the replicase-nascent chain complex of a replicating DI RNA could copy back on itself starting near the 3′-end of the nascent chain to give rise to snap-back molecules with inverted repeat sequences.

In addition to the unique origin proposed for DI_{011} RNA, yet a third model was suggested by *Keene* et al. (1981a, b) to account for the origin of stems in the unusual VSV DI-LT_2 (also called *DI 0.50*, see Figs. 1, 2). This model proposes the same specific promoter sequence recognition for resumption of synthesis, but differs from earlier models in that template switching occurs when a nascent minus strand terminates at residue 70, and resumes copying starting at the exact 3′-end of a second minus-strand genome template.

A complementary copy of this newly generated molecule would thus correspond to a genome-size minus strand with a 70-bp stem structure. The deletion event is viewed as a separate process taking place either before or after the template switch just described. The critical difference here is that resumption of synthesis occurs at the opposite end of a minus strand and involves the very terminal residue rather than an internal site. The origin of the uncoded extra A residue in position 71 is not explained but, as pointed out by the authors, the VSV polymerase is known to insert noncoded A residues in transcripts (*Colonno* and *Banerjee* 1978a; *Herman* et al. 1978).

In summary, the origin of terminal sequence complementarity in both VSV and Sendai DI RNAs is generally thought to occur by some sort of strand-switching event during polymerization. The essential features of this concept originate from the Kolakofsky-Huang copy-back model. However, attempts at explaining specificities in terms of sequence at resumption sites of synthesis, or in terms of particular strand selection, suggest that the models proposed so far may be too restrictive conceptually. Different mechanisms are perhaps involved in generating individual DI RNA species such as DI_{011} (= C5 ST) and DI-LT_2 (= *DI 0.50*) as opposed to other DI RNAs. However, it is probably more useful to consider a more general model which encompasses all structural features observed so far in all virus DI RNAs (see Section 4.3.3).

4.3.2 Genesis of Internal Deletions and Complex Genome Rearrangements

Internal genome deletions are now known to exist in DI RNAs of both negative- and positive-strand viruses as well as double-strand viruses (see Section 3). This type of genome rearrangement is thus observed more commonly than copy-back sequences which so far are restricted to rhabdo- and paramyxoviruses where they clearly predominate. This situation perhaps reflects unique polymerase and/or template properties of the latter virus groups.

One possible mechanism for the generation of internal deletions in DI RNA is that of splicing by host cell enzymes involved in the synthesis of mRNAs. The majority of animal RNA viruses, however, replicate exclusively in the cytoplasm. Nevertheless occasional accessibility of viral RNA templates to such enzymes (perhaps during cell division or after virus induced damage to the cell nucleus) cannot be ruled out. As mentioned previously (Section 3.3.2) the sequences flanking the simple internal deletions in the one influenza DI RNA examined bear no homology to the eucaryotic "consensus-type" splice sequences. The extensively rearranged SFV DI RNA also mentioned before (Section 3.4.1) also lacks such sequences at the recombinant sites (*Pettersson*, personal communication). It seems likely then that RNA viruses have evolved a "splicing mechanism" (breakage and ligation of RNA molecules) of their own, or rely on other mechanisms to achieve similar results. It is interesting to note here the parallel between genesis of internal genome deletions in DI RNAs and the long-standing and unresolved question of the mechanism responsible for genetic recombination of the nonsegmented picornaviruses genome (see discussion in next Section).

Only a few brief mentions of a possible involvement of a breakage-and-reunion, or "splicing" scheme, in the generation of internal deletions in DI RNAs have appeared in the literature (*Cole* 1975; *Lundquist* et al. 1979; *Kang* 1980; *Clerx-Van Haaster* et al. 1980). On the other hand, several authors suggested models which involve some sort of transcriptive or replicative error during viral RNA synthesis. These include: "copy-choice" of

RNA fragments (*Cole* 1975), or undefined templates (*Lundquist* et al. 1979) in poliovirus; recombination during transcription of alphavirus RNAs whose ends might be juxtaposed by virtue of terminal sequence complementarity (*Kennedy* 1976); replicase fall-off and reattachment at a distal point in the alphaviruses (*Guild* and *Stollar* 1977); similar resumption of synthesis at a specific site homologous to the VSV promoter sequence (*Schubert* et al. 1979); dissociation and reassociation of ScV (virus-like particle of yeast) transcriptase at some internal sequence (*Kane* et al. 1979); and looping out of the template and copying across a replication fork for VSV (*Kang* 1980). All of these suggestions are obviously variations on the same theme and reflect the preference of most authors for DI RNA generation based on "errors" during polymerization. Only the model of *Schubert* et al. (1979) suggests a sequence specificity in the resumption site of synthesis. A possible specificity in the fall-off sites for VSV polymerase (a short purine-rich stretch) was also suggested (*Keene* et al. 1980). Detailed sequence information on various DI RNAs is likely to accumulate very rapidly in the next few years (or even by the time this review is published). As in the case of procaryotic recognition sequences, the pattern of specificities if any may only become apparent by comparison of a large number of putative sites.

A more complex type of DI RNA rearrangement is the case of VSV DI *0.50* (= DI -LT_2) discussed in Section 3.2.4 (see also Figs. 2, 3). This DI RNA was most probably generated by two separate and distinct events, one resulting in an internal deletion and the other in a copy-back sequence (*Perrault* and *Semler* 1979; *Keene* et al. 1981a, b). The order in which these events might have occurred is interesting in this case because a VSV DI RNA with a simple internal deletion only was shown to possess very different biological properties (see Sections 5.2 and 5.4.1).

Multiple internal deletions (two or three) in the same DI RNA molecule were also reported in poliovirus and alphaviruses (see Sections 3.4.1 and 3.4.2). The heterogeneity in the position of the deletion in poliovirus DI is also interesting because it may reflect a relative lack of sequence specificity in the fall-off and resumption sites of synthesis within this particular region of the genome.

An additional instance of extensive rearrangement in a DI RNA is the peculiar case of the SVF DI examined by *Pettersson* and colleagues (Section 3.4.1). This particular rearrangement may not be representative of the majority of DI RNAs found in their heterogeneous mixture of species but nevertheless this information suggests caution in generalizing from the simpler DI RNA structures discussed so far, especially in togaviruses. Obviously, this SFV DI RNA has undergone multiple recombinational events most likely in succession. It is not immediately clear, how it acquired the tandem triplicate repeat structure or the various small inserts. However, if one assumes that the viral polymerase is responsible for this, then one is forced to hypothesize an even greater flexibility in template termination sites, as well as choice of strands and sequence specificity for resumption sites, than hitherto suspected. The general model of DI RNA generation presented in the next section does in fact attempt to remove many of the constraints inherent in previous proposals.

4.3.3 General Model

Several of the ideas advanced by many different workers regarding DI RNA generations can be incorporated in a more general view of these mutational events. Central to this view is the concept of promiscuous fall-off and resumption of synthesis by a viral poly-

merase-nascent chain complex. The copy-back model of Kolakofsky and Huang, for example, which first captured the essence of DI RNA generation, can then be viewed as one of many possible ways this process can take place. Stems, snap-back RNAs, internal genome deletions, repeats, inserts, and other rearrangements yet to be discovered, can all be accounted for by what can be called, the "leaping replication complex model" (see Fig. 5). Furthermore, an extension of this model also suggests a mechanism for recombination between homologous standard virus RNAs (see below.).

The essential features of the "leaping replication complex" model are as follows:
1. *Interrupted synthesis* – the generation of a recombinant site in DI RNAs (bringing together previously noncontiguous regions of a genome) does not involve ligation but interrupted synthesis of a plus or minus strand by the viral polymerase complex followed by resumption of synthesis at a different site.
2. *Variable choice of secondary templates* – the resumption site can be on the same template strand, or on the nascent chain carried by the leaping polymerase complex, or perhaps less frequently on a different viral template RNA which is not part of the original replicating complex.

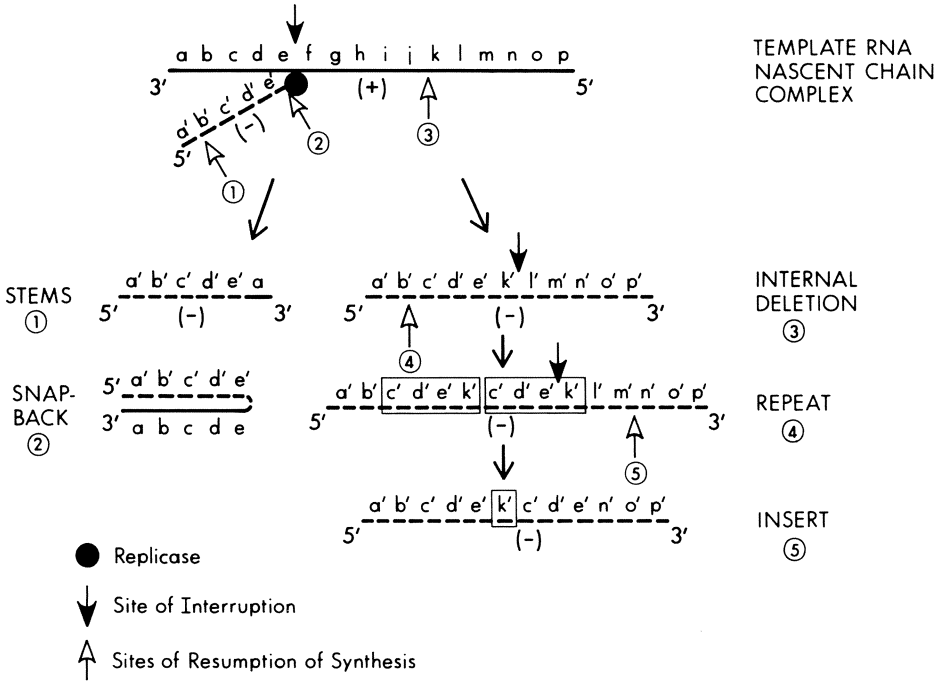

Fig. 5. A general model for the generation of DI RNA genome rearrangements. Five distinct types of minus-strand genome rearrangements are illustrated. The first two correspond to copy-back synthesis (see Fig. 4) resulting in either simple stems or snap-back molecules (see text, Section 4.3.1). The three other types of genomic rearrangements are illustrated on the right side of the figure as a succession of events (only the resulting minus strands are drawn for simplicity). Resumption of synthesis downstream from the interruption site leads to an internal deletion whereas resumption upstream produces a repeat sequence. A second deletion event results in an apparent insert sequence (k') flanked by repeats (c' d' e'). Further deletion event(s) can also result in apparent inserts not flanked by repeat sequences (not shown)

3. *Specificity of sites on secondary templates* – the viral polymerase-nascent chain complex can resume synthesis at variable sites of a secondary template (or the same template) with specificities and frequencies determined both by base sequence and viral nucleoprotein template properties (the contribution of each depending on the particular virus system).

As shown diagrammatically in Figure 5, resumption of synthesis on the nascent chain near its 5′-end, generates copy-back DI with small stems, while resumption near its 3′-end gives rise to snap-back RNAs. Similarly, resumption of synthesis on the same template strand further downstream from the termination site produces an internal deletion, whereas leaping back to a site upstream from the termination gives rise to a repeat sequence. Furthermore, an apparent insert sequence flanked by repeats can easily be generated starting from a simple deletion followed in succession by a repeat event and a further deletion event. The model is drawn in Figure 5 with a nascent minus strand only for the sake of simplicity. However, in some or all virus systems the leaps could occur within any replicating viral RNA complexes, DI or standard, regardless of polarity (but of course within the confines of the 5′- to 3′-rules of synthesis). Moreover, the model can also be drawn with circular replicating complexes and/or with more than one nascent chain per template. A leap from one replication complex to a different template not involved in the original termination event has also been suggested for VSV *DI 0.50* (see Section 4.3.1), although in this case the structure can equally well be explained by a copy-back event following complete reading of an internally deleted DI RNA template.

It is obvious that, in its most general form, the "leaping replication complex" hypothesis allows for an immense variety of structural rearrangements. It emphasizes the rather promiscuous abilities of viral replicase complexes to terminate and resume synthesis at a large variety of sites, in contrast to specifying events unique to the generation of each DI RNA molecule. The model does make one clear prediction, i.e., all DI RNAs must conserve at least one end sequence of the standard genome corresponding to the segment copied before interruption of synthesis. It should be remembered, however, that only those molecules capable of accumulating at the expense of standard viral RNA templates, i.e., possessing both the ability to replicate and interfere with standard virus synthesis, qualify as DI RNAs. Thus, the origin of these molecules is intimately tied to a discussion of the minimal template requirements for preferential replication (see Section 5.3, 5.4, and 5.5).

Before leaving the subject of the promiscuous leaping polymerase complex, I should point out that if one or more leaps occur symmetrically between two standard virus RNA molecules of the same polarity, but carrying different point mutations, the result is homologous genetic recombination in the classical sense (copy-choice scheme). Recombination frequencies as high as about 2% between point mutations on the same RNA molecule (as opposed to genetic reassortments between separate segments) can be observed in picornaviruses (*Cooper* 1977; *Romanova* et al. 1980) but have remained undetected in other RNA viruses. It seems likely that such recombination does indeed occur with all true RNA viruses but its frequency may be easily masked by high levels of spontaneous mutations (*Domingo* et al. 1978; *Semler* and *Holland* 1979; *Flamand* 1980; *Bruenn* and *Brennan* 1980; *Nottay* et al. 1981), frequent reversion at other sites (*Ramig* and *Fields* 1979; *Murphy* et al. 1980), and high levels of gene product complementation in polyploid particles (*Pringle* 1977). It is interesting to note that a scheme very similar to the leaping polymerase complex model described above was previously entertained as a possibility to explain genetic recombination in picornaviruses (*Cooper* 1977).

5 Replication and Interfering Properties of DI RNAs

5.1 Host Cell Modulation

Many of the studies discussed in relation to possible host effects on the de novo generation of DI (Section 4.1) can also be interpreted in terms of influence on DI replication and/or interference. I will discuss here only selected aspects of the previously mentioned reports as well as some additional studies which more directly implicate host control over DI amplification.

Perrault and *Holland* (1972a) suggested the existence of "low vs high interference" cells with regard to VSV DI replication. The basis for this distinction was suggested by the observation that VSV DI (from BHK_{21} cells) interfere much more strongly with standard virus replication in some hosts, such as BHK_{21}, HeLo, and chick embryo fibroblast (CEF) cells, versus others such as MDCK, PK-15, and MDBK cells. This variable interference potential of DI was also shown to be independent of their replication since they did replicate well in the "low interference" cells. A host cell factor(s) modulating the relative affinities of the viral replicase for standard and DI template was suggested as a possible explanation.

Huang (1977) also reported that VSV DI interference is stronger in fibroblast cells from young as opposed to old mice. This observation, which may be related to the ease with which VSV and rabies DI are produced in newborn versus adult mouse brain (*Holland* and *Villarreal* 1975) further suggests that the state of cell differentiation may affect DI interference.

The study of *Holland* et al. (1976a) also uncovered a different host cell effect on DI function. In this case, VSV DI (from BHK_{21} cells) strongly interfered with standard virus replication in HeLa cells but without concomitant replication. This result implies that DI replication per se is not necessary for expression of interference. As discussed previously, however, this same HeLa cell line does not always behave in this way (see Section 4.1). The same report from Holland's laboratory, as well as others (*Lunger* and *Clark* 1977; *Kang* et al. 1978a) also demonstrated that different host cells selectively amplify a different array of VSV DI from a VSV stock already containing seed quantities of DI. This result emphasizes the intricate relationship between replication of particular DI and putative host cell factors.

Turning to other virus systems, we can also find evidence for host cell modulation of DI amplification. *Crumpton* et al. (1981) very recently reported the selection of a different array of avian influenza DI RNAs in CEF versus BHK cells when starting with the same CEF-grown virus inoculum. Surprisingly, when the CEF-grown virus is switched to mouse L cells no change occurs in the DI RNA pattern but virion segment 6, and to a lesser degree segment 5, are reduced. The significance of these results is unclear since host factors related to permissiveness for viral growth may also be involved.

In the togavirus system, *Levin* et al. (1973) reported that SFV DI produced in CEF cells display more interference in mouse cells than in the cell of origin. Similarly, SV DI from BHK cells do not interfere or replicate in *Aedes albopictus* cells (*Stollar* et al. 1975; *Eaton* 1975; *Igarashi* and *Stollar* 1976). *Kascsak* and *Lyons* (1978) reported that Bunyamvera virus DI (from BHK_{21} cells) show stronger interference in BHK_{21} and Vero cells than in MDBK or *A. albopictus* cells. The same type of phenomenon was also reported for BHK_{21}-grown mengovirus DI which interfere more strongly in HeLa than BHK_{21}

cells (*McClure* et al. 1980). Similarly LCM virus DI interfere more strongly in BHK than in MDCK cells (*Dutko* and *Pfau* 1978).

The studies cited above strongly support the view that DI replication and/or interference with standard virus is subject to host control. A recent study by *Gillies* and *Stollar* (1980b), however, illustrates additional complexities in the interaction of DI with host cells. When VSV DI produced in *A. albopictus* cells are tested for interference with standard virus growth, they do so only with standard virus growing in the mosquito cells but not in BHK cells. In contrast, BHK-grown VSV DI interfere equally well with standard virus in both BHK and *A. albopictus* cells. When each DI preparation is grown for one additional passage in the heterologous cell types, the pattern of interference is reversed. These results strongly suggest that DI particles can be phenotypically modified by the host cell in such a way as to influence their interference properties. It is interesting to note that the earlier studies cited above may also involve undetected phenotypic alterations. The biochemical nature of this host modification(s), however, remains unclear.

How the host cell affects DI RNA replication specifically is unknown. Standard virus RNA replication is undoubtedly affected by the host cell as illustrated by the many instances of intracellular restriction of virus RNA replication, as well as the occurrence of host range mutants. The host cell could exert a direct influence on the viral RNA replicase, for example, by providing subunits as in the RNA bacteriophages (*Blumenthal* and *Carmichael* 1979). In addition or alternatively, a more indirect influence, such as membrane attachment sites, or availability of substrates, could be hypothesized. Progress in understanding the role of the host cell is likely to be dependent on the future availability of in vitro systems of replication for animal RNA viruses.

5.2 Transcriptional and Translational Activities of DI RNAs

Only two RNA virus DI systems, VSV and poliovirus, have been studied in detail with regard to transcriptional and translational activities. The lack of such activities in most VSV DI is well documented, and is clearly a result of their particular genome sequence rearrangements. In contrast, some poliovirus DI RNAs are efficiently transcribed and translated giving rise to abnormal truncated proteins. The situation in other virus systems is less clear cut because of insufficient relevant data. I shall review here what is known in each of these virus systems since the presence or absence of such activities may be biologically important for DI function.

The transcription of the VSV genome is a highly regulated process which is only partially understood. The five genes are seemingly transcribed by the VSV polymerase as if they were part of a single transcription unit beginning exactly at the 3′-end of the genome. The mandatory order of transcription is also accompanied by an attenuation process which results in stepwise decreasing amounts of gene transcripts as a function of their distance from the 3′-end of the template. It is not clear whether the transcriptase can only begin synthesis at this end of the molecule. In any case, it first gives rise to a 47-nucleotide long, plus-strand leader RNA of unknown function, and then probably continues with a separate initiation or processing event for each gene as it travels down the template. An alternative possibility is that initiation of each gene occurs simultaneously giving rise to very short transcripts, which are then elongated in the mandatory order (*Banerjee* et al. 1977; *Banerjee* 1980; *Testa* et al. 1980). Whatever the mechanism, it is clear

that a regulatory function must somehow subvert this transcription process, at some point in time during the virus life-cycle, and allow a polymerase to copy the entire template into a plus strand to serve as an intermediate in replication.

Turning now to VSV DI RNAs it is easy to see that, except for the unique class V DI all such templates lack the standard genome 3′-end sequence at their own 3′-end (see Fig. 2). Furthermore, the first three DI classes (the most common VSV DI) lack a sequence corresponding to the start of any gene. In retrospect, it is not surprising that *Huang* and *Manders* (1972) and *Perrault* and *Holland* (1972b) both reported lack of transcriptase activity in VSV DI both in vitro and in vivo. Likewise, it is also easy to see why the class V DI (*DI 0.46* = DI-LT_1) synthesizes four of the five VSV gene transcripts (missing L gene) both in vitro (*Colonno* et al. 1977) and in vivo (*Chow* et al. 1977; *Johnson* and *Lazzarini* 1977b). Moreover, these transcripts are translated into the corresponding VSV proteins. Perhaps more interestingly from the standpoint of the mechanism of VSV transcription, the class IV DI (*DI 0.50* = DI-LT_2) is transcriptionally inactive in vitro (*Perrault* and *Semler* 1979; *Keene* et al. 1981a, b) and would presumably be so in vivo as well. This latter finding underlines the importance of a correct "promoter" sequence at the very terminus of the template since this same sequence is found internally in *DI 0.50* but remains functionless.

From the above discussion, and remembering the rule that DI particles contain the same proteins as standard virions, it is not surprising to find that transcriptionally inactive VSV DI nevertheless contain a polymerase activity which synthesizes mRNAs when added to standard virus ribonucleoprotein templates (*Emerson* and *Wagner* 1972). What is more surprising, however, is that the 3′-end of these DI RNAs serves as template for the synthesis of a small 46-nucleotide long, minus-strand leader RNA in vitro (*Reichmann* et al. 1974; *Emerson* et al. 1977; *Semler* et al. 1978; *Schubert* et al. 1978) and in vivo (*Rao* and *Huang* 1979; *Leppert* et al. 1979; *Leppert* and *Kolakofsky* 1980; *Rao* and *Huang* 1980). The significance of this short RNA, which is apparently synthesized abundantly in vivo by the transcriptase rather than the replicase (*Rao* and *Huang* 1980) is unknown (see Section 5.4.1). Perhaps the availability of the VSV core protein N changes RNA synthesis from transcription to replication by binding to such small RNAs (N acting as antiterminator) as suggested by *Leppert* et al. (1979).

The unique transcribing ability of VSV *DI 0.46* (= DI-LT_1) offers several additional insights regarding VSV DI interference and replication (see Section 5.4.1). Suffice it to say here, that although this type of DI could play an expanded role in virus infections because it can complement deficient standard virus functions (*Johnson* and *Lazzarini* 1980) and unlike other VSV DI it can kill host cells (*Marcus* et al. 1977), it may only accumulate under rare circumstances since it is very easily outcompeted by the production of other nontranscribing DI, at least in BHK_{21} cells (*Perrault* and *Semler* 1979; *Epstein* et al. 1980).

It is important to note here that *Marcus* and *Sekellick* (1977) also propose that snap-back type VSV DI possess the unique ability (not shared by other VSV DI) to induce interferon very efficiently in host cells in the absence of standard virus. The same authors later proposed that such snap-back VSV DI may play a role in the establishment of persistent viral infections (*Sekellick* and *Marcus* 1978). However, other studies indicate a lack of correlation between snap-back VSV DI RNA and interferon induction (*Frey* et al. 1979). Very recently, *Frey* et al. (1981) presented evidence which shows that the variable interferon-inducing capabilities of VSV DI preparations can be directly correlated with small amounts of standard virus contamination.

We can now turn to a brief discussion of poliovirus DI RNAs which, in contrast to VSV DI, appear to be commonly transcribed and translated. These features were uncovered by Cole and colleagues in their initial characterization of poliovirus DI (see review by *Cole* 1975). It should be noted here that the distinction between transcription and replication in picornaviruses is somewhat blurred since the virion RNA and the mRNA are identical in sequence and differ only in that virion RNA contains a 5′-end covalently linked protein, VPg (see review by *Wimmer* 1979).

The ability to separate pure poliovirus DI from their standard virus (by density gradient centrifugation) enabled *Cole* and *Baltimore* (1973a) to test whether such DI can carry out any intracellular functions by themselves. Indeed, the poliovirus DI display all activities normally found for standard virus (similar rates of viral RNA and protein accumulation) except for a deletion of about one-third of the capsid-protein precursor which prevents them from packaging their RNA (*Phillips* et al. 1980). The shortened capsid precursor polypeptide is unstable and is rapidly degraded in infected cells. *Lundquist* et al. (1979) and *Humphries* et al. (1979) observed a very similar situation with their preparation of poliovirus DI. As mentioned previously (see Section 3.4.2) subsequent work showed that the DI RNA conserves the site for initiation of the polyprotein precursor (as well as the additional initiation site of unknown function) found in the standard genome (the exact position of this putative site is still unclear). As pointed out by *Lundquist* et al. (1979), the heterogeneity in the position of the DI RNA internal deletions could conceivably mask the presence of nontranslatable (out of reading frame) molecules. Nevertheless, these authors argued that the poliovirus polymerase may be a cis-acting protein early in infection (due to initial compartmentalization of parental genomes) and therefore only deleted RNA molecules capable of synthesizing a functional polymerase may be amplified. However, earlier experiments of *Cords* and *Holland* (1964) have clearly shown that poliovirus RNA can be readily replicated by a heterologous picornavirus polymerase supplied very early before significant multiplication of the poliovirus template. Since all picornavirus DI deletions so far mapped were all obtained from one particular isolate of poliovirus, there appears to be little basis for predicting that all such deletions should correspond to sequences coding for capsid proteins.

Surprisingly little information is available regarding possible transcriptional and translational activities of togavirus DI RNAs. The situation is somewhat more complicated than the other DI systems described so far because: 1. these are two classes of transcripts in standard virus infections, the full-size genome (indistinguishable in structure so far from the replication product) and the 26S transcript corresponding to the 3′-end one-third of the genome; and 2. togavirus DI RNAs vary greatly as to which genome sequences are deleted (mostly internal deletions). Nevertheless, as in the poliovirus DI system it would seem possible in many cases that togavirus DI RNAs, which are capped and polyadenylated (see Section 3.4.1), conserve a functional initiation site for protein synthesis, in particular the polymerase precursor site, near the 5′-end of the standard genome (the exact position of this site is not yet known). Aberrant nonfunctional proteins large or small, could then be produced and possibly degraded rapidly as in poliovirus DI. Aberrant proteins related to capsid precursor, however, may be much less frequent or nonexistent because the translation initiation site for these is normally cryptic in the full-size genome and is only active in the 26S transcript. Alternatively, the lack of a functional translation initiation site in togavirus DI RNA, may be essential for their interfering property (see Section 5.5).

A few instances of novel uncharacterized proteins in togavirus standard and DI coinfected cells were reported (*Weiss* and *Schlesinger* 1973; *Weiss* et al. 1974; *Guild* and *Stollar* 1975), but as suggested by *Weiss* et al. (1974), these could represent normal precursor molecules or by-products which for unknown reasons accumulate under conditions of interference only. The latter study also presented evidence that their 20S SV DI RNA is not translated in vitro and is probably not associated with polysomes intracellularly. A later study by the same laboratoy showed that no viral translation products can be detected by immunofluorescence techniques in cells infected with SV DI only (*Schlesinger* et al. 1975). *Bruton* et al. (1976) also reported that purified SFV DI are unable to carry out any step in virus multiplication except uncoating, and that SFV DI RNAs are not present on polysomes in coinfected cells. These clearly represent only a few attempts at detecting aberrant transcripts or translation products in togavirus DI infected cells. A more critical search for such products may well turn out to be useful in delineating control regions on the viral templates.

The situation in influenza virus DI RNA has been even less explored with regard to gene expression. The difficulties in obtaining pure DI populations were mentioned previously (Section 3.3.2). In addition, the segmented nature of the genome raises the fascinating question as to how the replication of one or more DI RNA segments, originating from one or more of the P genes, can so profoundly affect the replication of all segments. As in our previous discussion of togavirus DI RNAs, it seems plausible to suspect that a simple internal deletion might allow the transcription and possible translation of the shortened RNA. Aberrant transcripts and/or translation products related to DI RNAs have not yet been looked for in influenza-infected cells. Likewise, no information on this question is available for the segmented reovirus DI system. Pure reovirus DI, missing the L1 segment, were reported to transcribe the remaining nine segments normally both in vitro and in vivo (*Spandidos* et al. 1976). The remaining segments in reovirus DI can function in vivo to provide complementing functions (*Spandidos* and *Graham* 1975) and can undergo genetic reassortment (*Ahmed* and *Fields* 1981). *Bean* and *Simpson* (1976) also reported that influenza DI preparations contain somewhat reduced levels of transcriptase activity (not enough to account for the much greater loss of infectivity) but that all eight virion segments are transcribed. In the light of the more recent results regarding small DI RNAs in influenza and possibly reovirus, it would be rewarding to reexamine this question and determine whether these molecules are active as templates for transcription.

In summary, relatively little is known about gene expression in DI RNAs. This situation reflects the emphasis of past studies on VSV DI which are for the most part genetically inert. This certainly indicates that RNA viruses are not forced to rely on such gene expression from their DI templates in order to achieve interference and control over their own multiplication. Nevertheless, gene expression in DI RNAs of some viruses may be common perhaps without playing a significant role. Such aberrant gene expression in naturally occurring deletion mutants could prove to be useful in elucidating control functions.

5.3 Conserved Replication and Encapsidation Origins

All DI RNAs examined to date conserve one or both end sequences of the standard virus genome. This feature is central to the models for the origin of DI RNAs discussed

previously (Section 4.3) and is not unexpected since all true RNA viruses most likely replicate using a virus-coded polymerase initiating synthesis at the very 3′-OH end of viral template molecules. Thus the 3′-end sequences of both plus and minus RNA strands must correspond, at least in part, to replication origin signals (the sequences complementary to these can also be viewed as termination signals although viral RNA replicases could simply continue synthesis to the very end of the template without regard to sequence specificity). In rhabdo- and paramyxoviruses, DI RNAs usually conserve the 3′-end sequence of the intracellular plus-strand complement of the standard genome at the 3′-end of both their plus and minus strands. This feature may in fact be responsible for their interfering properties (see Section 5.4.1 below). Internally deleted DI RNAs obviously conserve both ends. The only possible exception to this rule is the extensively rearranged SFV DI discussed previously (see Section 3.4.1) but this remains speculative until more information regarding the sequence at the 5′-end of the SFV standard genome is obtained.

Some tentative conclusions regarding the minimum size of conserved replication origins can be gathered from DI RNA base sequence data available so far. In VSV, the stem sizes are 45–70 bp long. Since the sequences beyond these recombination sites vary in different molecules (see Fig. 2) it is very likely that the replication origin signal for minus-strand synthesis does not extend beyond the first 45 residues at the 3′-end of the standard genome plus-strand template. The larger stem sizes in Sendai virus (110–150 bp) could conceivably reflect a longer signal sequence, but other explanations, such as a more distally located encapsidation signal (see below) can also be hypothesized. The only influenza DI RNA sequenced to date contains an internal deletion located very near the center of the virion segment of origin (Section 3.3.2) and thus little can yet be said regarding the minimum sequence needed for replication. The SFV DI RNA previously mentioned contains a conserved genome 3′-end sequence segment 106 nucleotides long beyond the poly A tail. It is not clear whether this noncoding segment represents a minimum size for a replication origin (perhaps in addition to the poly A tail) but it is intriguing to note that reverse transcription of the 3′-end of the SFV standard genome shows a very strong stop at residue 104, and a similar stop (not quite as strong) is also found for SV at position 145 (*Ou* et al. 1981). The secondary structure of the template at these positions (perhaps involving partial complementarity with the 5′-end) may determine an important signal for replication. The pattern which emerges from these few examples is that only a relatively short 3′-end sequence (less than 150 nucleotides) is sufficient for recognition as a specific replication origin in RNA viruses. Rapid accumulation of sequence data for a number of DI virus systems in the near future should provide a more refined picture of the minimal requirements for such signals.

DI RNAs must obviously also conserve the sites necessary for encapsidation. A priori these sites could be anywhere on the standard virus genome and not necessarily at the ends. This is exemplified in the case of the plant tobacco mosaic virus RNA where the recognition site for initiating the assembly of its helical ribonucleoprotein is situated about 15% of the way from its 3′-end (*Otsuki* et al. 1977; *Zimmern* 1977). Nevertheless, as mentioned previously (Section 4.3.1) very short nascent strands of the VSV replication complex are rapidly assembled into a helical ribonucleoprotein structure. It is therefore very likely that a capsid protein recognition site is situated very near the 5′-end of the VSV genome. Likewise, VSV standard genome plus strands are always found encapsidated intracellularly and a similar site is probably present at the 5′-end of these plus strands (the

two 5′-end sequences are almost identical for the first 20 or so residues, see Fig. 2). Very recently, *Blumberg* et al. (1981) provided strong support for this idea since the small plus- and minus-strand VSV leader RNAs, corresponding to the first 47 and 46 residues at the 5′-ends of genome length molecules, are found in association with the VSV nucleoprotein in vivo. An analogous situation probably holds true for the paramyxoviruses which use a similar strategy for replication. The encapsidation sites for other RNA viruses, however, remain undefined except to the extent that DI RNAs must conserve them to be packaged.

Icosahedral RNA viruses (picornaviruses, togaviruses, reoviruses) appear to have packaging constraints influencing DI RNA selection and these may be more stringent than in helical symmetry viruses. It seems reasonable to speculate that, in addition to conserving a recognition site for initiating nucleocapsid assembly, the overall stability of DI particles must be equal or nearly equal to that of standard virus. In VSV, for example, DI containing RNAs as small as 10% of the genome are stably packaged. In togaviruses, on the other hand, standard size capsids appear to contain multiple copies of smaller DI RNA (1/6 to 1/2 of genome length) which together add up to an RNA content similar to standard virus (*Bruton* and *Kennedy* 1976; *Kennedy* et al. 1976; *Guild* et al. 1977; *Stark* and *Kennedy* 1978). Packaging restraints were also suggested to play a role in DI RNA selection in picornaviruses (*Cole* 1975; *Lundquist* et al. 1979; *McClure* et al. 1980; see also Section 3.4.2). It is possible that specific viral RNA sequences other than the site involved in the initiation of nucleocapsid assembly are in some cases necessary for the construction of biologically stable and functional DI particles.

5.4 Interference Mechanisms of VSV DI

5.4.1 Replicase Competition and Terminal Sequence Complementarity

There are as yet no unifying hypotheses which can explain interference mechanisms by DI of all RNA viruses. This is perhaps not surprising since different RNA virus groups display quite unique strategies for viral replication. Even in the best studied VSV DI system there are probably two distinct interference mechanisms corresponding to two different types of DI and both of these are still poorly understood.

Several years ago, *Huang* and *Baltimore* (1970) suggested that interference by DI might be analogous to the successful replication competition by short fragments of Qβ bacteriophage RNA with full-length RNA during in vitro synthesis (*Mills* et al. 1967). However, it was soon realized that VSV DI RNAs do not compete by virtue of size since the smallest DI are not invariably selected during replication (*Perrault* and *Holland* 1972a; *Holland* et al. 1976a). Moreover, a similar specific interfering ability was observed for all VSV DI regardless of size (*Reichmann* and *Schnitzlein* 1977; *Adachi* and *Lazzarini* 1978). Nevertheless smaller VSV DI RNAs can conceivably be favored for replication over long-term passage in some cases. This phenomenon is quite distinct from the overwhelming and quantitatively similar replicative advantage displayed by different size VSV DI RNAs in a single replication cycle (see also Section 5.4.2 below for a discussion of interference based on size in other RNA virus DI).

Studies by *Stampfer* et al. (1969), *Perrault* and *Holland* (1972b), and *Huang* and *Manders* (1972) established that VSV DI RNAs not only preferentially replicate but are

incapable of transcription on their own and do not inhibit primary transcription by input parental virus. Overall reduction of secondary transcription and viral translation products was proposed to result indirectly from inhibition of genome RNA synthesis. These observations led to the suggestion that VSV DI RNAs successfully compete for the VSV replicase by virtue of a higher affinity for the enzyme. In addition, it was proposed that DI RNAs can gain a kinetic advantage in replication by virtue of their availability as templates while the standard genomes are engaged in active mRNA transcription. These general concepts were later strengthened by further quantitative studies of RNA species in standard and autointerfered infections (*Palma* et al. 1974; *Stamminger* and *Lazzarini* 1977) and still remain to date the most probable explanation for interference by the majority of VSV DI. More recently, the discovery of the unique structural property of VSV DI RNAs represented by stems led *Perrault* et al. (1978) to suggest that this particular feature plays a central role in interference.

According to this proposal, the replication origin at the 3′-end of the VSV genome minus strand is recognized by a different replicase than the one playing a similar role at the 3′-end of the plus strand. The two types of replicases were further postulated to differ by the presence or absence of host cell factors. The synthesis of plus and minus DI RNAs can thus be accomplished by the same replicase (the one responsible for minus-strand genome replication) and involves only one kind of recognition event rather than two for standard virus. Furthermore, an additional kinetic advantage is gained if the synthesis or availability of plus-strand genome templates is rate-limiting during standard virus replication. A slightly different model involving only one replicase recognizing a "high affinity" binding site at the 3′-end of standard genome plus strands and a "low affinity" binding site at the 3′-end of standard genome minus strands was suggested by *Huang* et al. (1978). It is also possible to view the preferential replication of these DI RNAs (without invoking differential binding affinities) as a mass effect resulting from two DI RNA templates, with identical 3′-terminal sequences, competing with one standard virus RNA template for a single type of replicase (*Reichmann* and *Schnitzlein* 1979).

These models, which are not mutually exclusive, necessarily remain very speculative at this time since very little is known regarding the mechanism of standard VSV RNA replication. Nevertheless, the importance of stems in governing the replicative advantage of VSV DI RNAs is strongly supported by the observation that the transcribing VSV *DI 0.46* (= DI-LT_1), which lacks such complementary end sequences (see Fig. 2), replicates very poorly in the presence of other stem-containing DI RNAs during a single cycle of infection (*Perrault* and *Semler* 1979). This is so even when compared to *DI 0.50* (= DI-LT_2) which aside from stems differs only in having a slightly longer 5′-end genome segment. The transcribing VSV *DI 0.46* alone however, appears to interfere as well with standard virus replication as other VSV DI (*Reichmann* and *Schnitzlein* 1977; *Adachi* and *Lazzarini* 1978; *Reichmann* and *Schnitzlein* 1979). Whatever the mechanism of interference might be for this unique *DI 0.46* (see below), it appears to be superceded by the replicative advantage conferred by stems. It is likely also that the mere presence of these end sequences prevents any DI RNA from functioning as a template for mRNA synthesis. This is exemplified by VSV *DI 0.50* which is transcriptionally inert even though it contains all sequences necessary for transcription of four VSV genes (see Sections 3.2.4 and 5.2). This lack of transcription as mentioned above, could be crucial in governing interference.

Aside from preventing transcription, VSV DI stem sequences also serve as template

for the synthesis of the short minus-strand leader RNA 46 nucleotides long) in vitro and in vivo (see Section 5.2). This small RNA is found only in very low levels in standard virus-infected cells, but is synthesized in 50-fold or more molar excess over DI RNA template in interfered infections (*Rao* and *Huang* 1979; *Leppert* et al. 1979; *Leppert* and *Kolakofsky* 1980; *Rao* and *Huang* 1980). The efficient synthesis of this minus-strand leader RNA from DI templates led *Rao* and *Huang* (1979) to suggest that it may play a role during interference by binding enzymes or substrates or by blocking specific complementary sequences. As mentioned previously (Section 5.2) a model suggested by *Leppert* et al. (1979) proposes that the availability of the VSV nucleocapsid N protein regulates the switch from transcription to replication by binding to the small plus-strand leader RNA (the 47-nucleotide long RNA copied from the 3′-end of the minus strand genome in vitro and in vivo) and promoting read-through synthesis of plus-strand genome copies past this strong stop signal. If the minus-strand leader RNA plays a similar role for minus-strand genome synthesis, then a decrease in the availability of N protein in interfered infection might account for the accumulation of the small minus-strand leader (*Leppert* et al. 1977; *Leppert* and *Kolakofsky* 1980). These speculations are consistent with the reported transcriptive mode of synthesis of this minus-strand leader (resistant to cycloheximide and enhanced in ts mutants which increase transcription) as well as its lack of association with the N core protein in interfered infections (*Rao* and *Huang* 1980). The latter is in contrast to plus-strand leader which for the most part is assembled into a ribonucleoprotein in standard virus infections (*Blumberg* et al. 1981). It is tempting to speculate that the plus-strand leader RNA synthesized in interfered infections might be rapidly degraded because of lack of N protein, thereby leading to decreased genome RNA synthesis. Whether this is the case or not, the continued study of these small leader RNAs, which represent initiating events in VSV replication, is at present a most promising approach to elucidating the details of DI-mediated interference and VSV genome replication.

As mentioned above, interference by the transcribing VSV *DI 0.46* most likely involves a different mechanism. Aside from the unique structural and replicative features already discussed, a number of additional properties of this DI support this notion. It is the only VSV DI shown to interfere heterotypically with the New Jersey VSV serotype (*Prevec* and *Kang* 1970; *Schnitzlein* and *Reichmann* 1976). The basis for this is unclear but the heterotypic interference is restricted to the Concan subgroup of VSV New Jersey and does not occur with the Hazelhurst subgroup (*Adachi* and *Lazzarini* 1978; *Reichmann* et al. 1978; *Metzel* et al. 1978; see also following Section).

The transcribing VSV DI also displays an unusual inactivation target size. A number of laboratories have shown that VSV DI possess a smaller UV target size for replication and interference than standard virus infectivity (*Holland* et al. 1976a; *Winship* and *Thacore* 1979; *Bay* and *Reichmann* 1979). In the careful quantitative study of *Bay* and *Reichmann* (1979) the target size of interference by various DI particles was found to correspond to the size of the whole molecule. The one exception, however, is the transcribing DI which exhibits a target size equivalent to about 42% of its RNA. In addition, these investigators showed that, in contrast to other VSV DI, the unique transcribing DI interferes with primary transcription of input standard virus in vivo. This DI isolate was shown previously to carry out its own in vivo primary transcription (minus the L gene) as well as some synthesis of DI size plus and minus strands in the absence of helper virus (*Johnson* and *Lazzarini* 1977b). Thus the unusual UV inactivation target size was inter-

preted as being a compromise between the requirements for replication of its genome and those for interference with primary transcription. These studies were recently further extended by *Bay* and *Reichmann* (1981) who demonstrated that active transcription in vivo by the DI itself is necessary for interference with heterotypic virus primary transcription and standard virus production. It is apparent from these results that the mechanism of interference by this unique DI is complex and involves at least one step of standard virus replication which is not affected by the interference activity of other VSV DI. Unfortunately at this time, the occasional preliminary reports on the isolation of new VSV DI originating from the 3'-end of the standard genome (*Adler* and *Banerjee* 1976; *Reichmann*, personal communication) which might be expected to behave like *DI 0.46* have not yet been confirmed.

5.4.2 Additional Parameters Affecting VSV DI Interference

Several additional aspects of interference by VSV DI need to be mentioned in order to appreciate the complexity of the phenomenon. Most importantly, replication of VSV DI RNAs is not always accompanied by interference. There are at least three distinct situations where this uncoupling can be observed: 1. growth of DI in "low interference" cell lines, 2. adding DI late during the course of standard VSV growth, and 3. support of DI with heterotypic helper virus.

The first of these situations has already been discussed in detail in Section 5.1. The second was noted by *Stampfer* et al. (1969) when they added VSV DI 2.5 h after infection with standard virus. This observation emphasizes that interference involves competition for an early step of virus multiplication (beyond penetration). In this regard it is interesting to note that VSV DI RNAs are rapidly assembled into nucleocapsids but accumulate under conditions of interference in the infected cell. They appear to be more slowly and less efficiently matured to virions than standard virus (*Palma* and *Huang* 1974; *Khan* and *Lazzarini* 1977; *Moyer* and *Gatchell* 1979). This deficiency of DI nucleocapsid budding may be due to insufficient availability of viral membrane proteins (*Moyer* and *Holmes* 1981) although what causes this protein specific deficiency is unknown.

The third case of replication without interference can be observed when Indiana VSV DI are supported by a heterotypic New Jersey VSV helper virus (*Schnitzlein* and *Reichmann* 1977b; *Khan* and *Lazzarini* 1977; *Adachi* and *Lazzarini* 1978). As mentioned before (previous Section) only the unique transcribing VSV Indiana DI causes interference under these conditions. These studies suggest that protein specificities, probably involving interactions between heterotypic polymerase and template ribonucleoproteins, are important in determining the outcome of competition between DI and standard genome RNA. The base sequence divergence between the two serotypes is extensive enough to result in very different T1 oligonucleotide maps (*Clewly* et al. 1977). The protein specificity of interference is further illustrated by the fact that chimeric DI (Indiana VSV DI RNA encapsidated in New Jersey VSV proteins) show a dramatic increase over pure Indiana DI in their interfering ability towards New Jersey standard virus (*Schnitzlein* and *Reichmann* 1977b).

Holland et al. (1976a) also reported a different sort of uncoupling phenomenon whereby VSV DI show strong interference in HeLa cells without apparent replication of biologically active DI ribonucleoproteins in the cytoplasm (pure DI RNA is not biologically active in interference assays). This exceptional case could either involve the repli-

cation of biologically inactive DI ribonucleoprotein (unable to interfere in BHK_{21} cells) or point to the existence of yet a different type of interference phenomenon in HeLa cells. The variable behaviour of HeLa cells in terms of DI replication mentioned previously (Section 4.1) however, hampers efforts to study this phenomenon further.

An additional important aspect of VSV DI interference is the question of whether it is an all or none phenomenon at the single cell level. The classical studies of *Bellett* and *Cooper* (1959) showed that, at a constant input multiplicity of standard virus, increasing input concentrations of DI result in an exponential decline of plaque-forming progeny in chick embryo cells at least over two orders of magnitude. These first-order kinetics at low-input concentrations of DI suggest that only one DI particle per infected cell is required to elicit complete interference. In contrast to this, *Khan* and *Lazzarini* (1977) reported the absence of single-hit inactivation with their VSV DI in BHK_{21} cells using radiolabeled viral RNA quantitation as their assay (massive replication of DI_{011} occurs at low-input DI multiplicities with only marginal interference with standard virus RNA synthesis). These contrasting results could be due to different host cells or virus strains employed, or the nature of the assays. More recently, *Sekellick* and *Marcus* (1980) analyzed plaque-forming virus yields from individual Vero-Green monkey kidney cells infected with standard VSV virus or DI. They concluded that indeed the presence of a single functional DI in a cell is sufficient to inhibit essentially all infectious virus production. However, at higher multiplicities of DI less interference is observed so that many coinfected cells yield intermediate levels of infectious virus. In light of the many parameters affecting DI generation, replication, and interference discussed above, it seems reasonable to expect that the all or none interference results obtained represent only one end of the spectrum governing the interaction between standard virus and DI.

A different approach to determining critical parameters in VSV DI interference was also pursued by *Schnitzlein* and *Reichmann* (1977a). These investigators compared nucleotide sequence homologies (by hybridization techniques) between one short DI RNA derived from the Glasgow New Jersey VSV isolate (now referred to as subtype Hazelhurst) and the Ogden New Jersey standard virus (now referred to as subtype Concan) with which it interferes efficiently. By this annealing test, the two RNAs are found to share no more than about 230 nucleotides in common (not necessarily in a single stretch). Similarly, the transcribing VSV Indiana DI which interferes heterotypically with New Jersey subtype Concan virus (see above) also shares only a small sequence, about 260 nucleotides long, in common with the heterotypic standard virus genome. These homologies are probably related, at least in part, to the highly conserved genomic end sequences of these different VSV serotypes as determined by direct base sequencing (*Colonno* and *Banerjee* 1978b; *Rowlands* 1979; *Keene* et al. 1979; *McGeoch* et al. 1980; *Keene* et al. 1981a). These data therefore also indirectly support a critical role for terminal sequences in the phenomenon of VSV DI interference.

Perhaps the most important instance of factors modulating interference by VSV DI, at least from an evolutionary point of view, is the situation documented by *Horodyski* and *Holland* (1980) and referred to briefly in Section 2.3. The standard VSV genome undergoes rapid and extensive mutational drift during persistence in cell culture or during repeated high multiplicity lytic passage in the presence of DI (*Holland* et al. 1979; *Rowlands* et al. 1980). As little as 34 days after initiation of virus persistence, the standard virus recovered in some cases is no longer subject to interference by the VSV DI originally employed to establish the persistent culture, and cannot act as a helper for their replication.

Clones from these standard virus mutants, however, can still generate "interfering type" DI de novo when passaged serially (*Horodyski* and *Holland* 1980). The basis of this phenomenon is undoubtedly related to the selection of standard virus mutations which specifically affect interference by VSV DI. *Semler* and *Holland* (1979), for example, demonstrated the occurrence of seven base substitutions in the first 46 nucleotides at the 5′-end of the standard genome after 5 years of persistence. These results once again emphasize the importance of the minus-strand genome replication origin in controlling interference by VSV DI since the genome 3′-end sequence mutates much less rapidly during persistence (*Rowlands* et al. 1980). Mutations affecting viral polymerase protein activity are also likely to occur under these conditions, and the coevolution of replicase and template RNA structures during persistence provides a novel and fascinating approach for the study of virus attenuation.

Lastly, a recent report demonstrates an additional unusual aspect of VSV DI interference in that one particular VSV DI (*DI 0.52*) invariably and efficiently outcompetes the replication of a smaller VSV DI (*DI 0.33* = DI-T) in a single cycle of replication (*Huang* et al. 1981). This observation, which is reminiscent of the situation with the unique transcribing VSV DI which is outcompeted by other VSV DI, is interesting because both DI RNAs in this case were generated independently from the same virus and correspond to type I structures (see Fig. 1). The stem sizes are 55 and 45 bp long for the larger and smaller RNAs respectively (*D. Rao* and *A.S. Huang*, personal communication). The basis for the replicative advantage of the larger DI is unknown but it is unlikely to be directly related to stem sizes since this parameter does not affect relative interfering ability in other VSV DI (see previous Section). The presence of different point mutations in each of these two DI RNAs which are not present in standard virus was also reported and is perhaps responsible for this odd behaviour (*Hagen* and *Huang*, 1981). The accumulation of point mutations in DI RNAs may be more widespread than hitherto suspected and may also be related to the phenomenon of mutational drift observed in standard virus RNA during DI-mediated virus persistence.

5.5 Interference Mechanisms of Other RNA Virus DI

The complexities relating to DI interference in VSV are certainly not unique to this virus. The absence of similar documentation in other RNA virus DI systems reflect the paucity of relevant studies. Except for togaviruses and picornaviruses virtually nothing is known regarding mechanisms of interference, other than the general concept of replicase competition. In some cases, such as host cell effects on interference (Sections 4.1 and 5.1), the results obtained are analogous to the VSV system. However, it is reasonable to suspect that interference mechanisms might reflect the differing replication strategies of each virus group. Moreover, in contrast to VSV and paramyxoviruses, the majority of DI RNAs in other viruses are internal deletions. The segmented influenza and reoviruses also present an intriguing problem since DI RNAs derived from specific gene segments may interfere with the "coordinate" replication of all segments.

Schlesinger et al. (1975) proposed a simple model for alphavirus DI interference based on straightforward competition for a limited amount of viral replicase synthesized by standard virus. *Bruton* et al. (1976), however, proposed a mechanism for DI interference in this virus group based on a more rapid synthesis of DI RNAs because of their

small size. Assuming that elongation rate might be limiting during RNA synthesis in vivo, the shorter DI RNAs, which contain the original genome replication origins at their ends, might be expected to accumulate faster as a result of two competition processes operating sequentially, one for the synthesis of minus strands and one for synthesis of plus strands. Some support for this hypothesis comes from the aforementioned sequential deletion phenomenon observed when togavirus DI are passaged repeatedly (see Sections 3.4.1 and 4.1). It is not clear, however, whether this evolution towards smaller size DI molecules involves the same mechanism as the one responsible for interference in a single replication cycle. It also seems unlikely that elongation rates rather than initiation rates would govern overall accumulation of viral RNAs in infected cells. More detailed studies of interference parameters in togavirus infections are needed to clarify these points. The different rates of selection of smaller DI RNAs upon passage in different cells (*Stark* and *Kennedy* 1978) do argue strongly that other factors besides size (host cell factors, encapsidation constraints, etc.) play an important role in interference by togavirus DI.

The UV target size for interfering activity by togavirus DI is smaller than that of virus infectivity (*Dimmock* and *Kennedy* 1978; *Kowal* and *Stollar* 1980) as found for VSV (Section 5.4.1) and influenza DI (Section 3.3.2). In the case of SV DI produced in BHK cells, the target size was shown to correspond to the physical size of the DI molecule but, oddly enough, that of SV DI produced in *A. albopictus* cells was about 25–30% smaller than expected (*Kowal* and *Stollar* 1980). It is not clear whether the latter case is due to complications resulting from the use of a different host cell in the assay or whether it suggests a different mechanism of interference as in the case of the VSV *DI 0.46* (Section 5.4.1).

Interference by poliovirus DI is thought to result from two independent effects acting in concert (*Cole* 1975). In contrast to other RNA viruses, the total level of viral RNA synthesis in cells coinfected with standard and DI remains essentially the same (this may reflect the relatively lower levels of interference seen in this system). The replication of standard virus RNA is thus decreased by a percentage close to the percentage of DI in the inoculum (as mentioned previously in Section 5.2, poliovirus DI synthesize RNA as efficiently as standard virus). Synthesis of capsid precursors coded for by the standard genome only is therefore reduced by a similar amount. A prediction borne out by the results obtained is that the percentage of standard virus produced in coinfected cells is the square of the percentage of standard virus in the inoculum. This mechanism, however, does not account for the enrichment or preferential replication of poliovirus DI (about 5–8% per passage in the studies of *Cole* and colleagues). This crucial phenomenon remains unexplained but can be affected by temperature and transient treatment with cycloheximide (*Cole* and *Baltimore* 1973c). Some possible explanations involving preferential encapsidation or replication of DI RNAs, or more rapid completion of the first round of protein synthesis in the earliest events of infection were suggested (*Cole* 1975). As in other virus DI systems, however, the interference phenomenon is likely to be complex since the interference level of another picornavirus (mengovirus), is affected by the host cell (Section 5.1).

Overall, it seems clear that a detailed understanding of interference by RNA virus DI is limited by our current knowledge of standard virus replication. Progress in one area is often spurred by advances in the other. The elucidation of the exact segment composition of infectious influenza virions, for example, provided the basis for the characterization of DI RNAs. On the other hand, the discovery of stem structures in VSV DI

RNAs is paving the way for understanding initiation of RNA replication in standard virus.

6 DI of DNA Viruses - Brief Overview

For reasons outlined in the introduction to this review, DI of DNA viruses may be thought of as playing a less important role in nature than DI of RNA viruses. It is intriguing, however, to note the many parallels between these two different worlds of defective viruses. All DNA viruses, with the possible exception of adenoviruses and poxviruses, have been shown to generate DI. The best studied are the so-called evolutionary variants in the papovaviruses (reviewed in *Fareed* and *Davoli* 1977; *Kelly* and *Nathans* 1977; *Brockman* 1977; *Fried* and *Griffin* 1977). These clearly interfere with standard virus replication by virtue of reiterated origins of replication (*Lee* and *Nathans* 1979). They contain extensive rearrangements of the viral genome which are often linked to host cell DNA sequences. Some specificity in the type of host DNA selected may exist perhaps in view of substituting for viral functions (*Papamatheakis* et al. 1981; *Carroll* et al. 1981).

Parvovirus DI often contain internal genome deletions and snap-back DNAs (*Faust* and *Ward* 1979; *de la Maza* and *Carter* 1980) similar in structure to some VSV DI RNAs (Section 3.2.3). The origin of these snap-back DI DNAs probably differs from their VSV counterparts, however, since parvovirus standard DNA replicates via self-complementary or snap-back intermediates. Herpesviruses also generate DI which contain extensive genome rearrangements (*Murray* et al. 1975; *Kaplan* et al. 1976; *Henry* et al. 1979; *Stinski* et al. 1979; *Frenkel* et al. 1980). Even the filamentatous DNA bacteriophages were shown to produce DI (*Griffith* and *Kornberg* 1974; *Enea* and *Zinder* 1975; *Chen* and *Ray* 1978; *Ravetch* et al. 1979).

Recombinational mechanisms leading to generation of DI genomes of DNA viruses may well have features in common with general recombination in eucaryotic cells. Replication of small DNA viruses in particular is very much dependent on the host cell DNA replication enzymes. Nevertheless, there may be unknown recombinational features unique to the virus replication complex which govern DI generation. The recurring and independent emergence of very closely related evolutionary variants of SV40 (with identical host-viral DNA sequence junctions) suggests that some unknown mechanism leading to nonhomologous recombination between viral and host genomes is at play (*Singer* et al. 1978; *Papamatheakis* et al. 1981).

A host effect on the generation and/or amplification of papovavirus DI (*Norkin* et al. 1981; *O'Neill* and *Carroll* 1981), reminescent of that observed in RNA viruses, was also reported. The most intriguing parallel between the two virus systems, however, is the accumulating evidence that DI of DNA viruses also play a role in the establishment and/or maintenance of persistent infections. This has now been reported for SV40 and BKV (*Norkin* 1979; *O'Neill* and *Carroll* 1981), human cytomegalovirus (*Stinski* et al. 1979), and equine herpesvirus type 1 (*Robinson* et al. 1980). Of course, DNA viruses can also persist in host cells by means of integration. Perhaps DI confer evolutionary advantages in certain types of host virus associations. Whatever the case may be, the potential biological role of DI in DNA viruses is worthy of further exploration.

7 Concluding Remarks

The last decade has witnessed a major expansion of studies focused on DI biology. The diversity of genomic sequence rearrangements in these particles reflects the increased plasticity and evolutionary adaptability of viral genomes when they are no longer required to code for an infectious unit. DI genomes most likely accumulate by selective amplification of some defective molecules generated at low frequency during standard virus replication. The genomic rearrangements in DI of RNA viruses can best be explained by strand-switching of the viral replicase complex during synthesis. Some DI RNA structures strongly suggest that this strand-switching can occur between different genome templates. The parallels between this mechanism and homologous genetic recombination (see general model, Section 4.3.3) are intriguing and imply strongly that such recombination is universal among RNA viruses.

DI effectively outcompete their parent helper virus for multiplication without suppressing their growth entirely. The various DI template structures therefore teach us the minimal sequence requirements useful in controlling virus multiplication, a lesson which suggests strategies for preventing virus disease in the future.

The host cell often affects the replication and properties of DI independently of its influence on standard virus and more importantly, the interplay between infectious virus, DI, and host cell often leads to virus persistence in culture without cell killing. It would be surprising if DI were not involved in at least some aspects of virus multiplication in animal and human infections. Measles virus can certainly persist in humans for several years before it triggers subacute sclerosing panencephalitis. Other chronic or degenerative human diseases are also suspected to involve slow growing or latent viruses. DI could play a role in such infections but this may be difficult to establish if they exert their effects locally or during a restricted phase of virus spread.

Perhaps more important than the disease aspect, DI represent an important evolutionary element in nature. These subgenomes represent ideal vehicles for rapid evolution since they are much freer to mutate than infectious virus. Moreover, they appear to exert a mutational pressure on the standard virus itself by virtue of their interfering properties. It is conceivable that DI are only a subclass of virus-related sequences which interact with both host cells and infectious viruses. In analogy to the intimate relationship of DNA virus genomes and transposons with host cells, one can also imagine RNA virus sequences (DI?) remaining in association with their host in an episomal form, after losing the ability to be packaged and transmitted to other cells. The virus-like particles of fungi may in fact represent such an association.

The next decade of research on DI is likely to reveal fascinating aspects of virus growth control. A great deal of sequence analysis, now a favorite tool of nouveau molecular biologists, is likely to shed some light on mechanisms of origin and replication of DI. The basic conclusion that replication origin sequences are a major determinant of interference by these particles will undoubtedly be refined by the advent of in vitro systems of replication. Lastly, continued study of the role of DI in mutational drift during persistence is setting the stage for a much more dynamic understanding of virus evolution.

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