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The Rule of Six, a Basic Feature for Efficient Replication of Sendai Virus Defective Interfering RNA

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The addition of the hepatitis delta virus genomic ribozyme to the 3′ end sequence of a Sendai virus defective interfering RNA (DI-H4) allowed the reproducible and efficient replication of this RNA by the viral functions expressed from cloned genes when the DI RNA was synthesized from plasmid. Limited nucleotide additions or deletions (+7 to −7 nucleotides) in the DI RNA sequence were then made at five different sites, and the different RNA derivatives were tested for their abilities to replicate. Efficient replication was observed only when the total nucleotide number was conserved, regardless of the modifications, or when the addition of a total of 6 nucleotides was made. The replicated RNAs were shown to be properly enveloped into virus particles. It is concluded that, to form a proper template for efficient replication, the Sendai virus RNA must contain a total number of nucleotides which is a multiple of 6. This was interpreted as the need for the nucleocapsid protein to contact exactly 6 nucleotides.

Sendai virus (SV) is a member of the *Paramyxoviridae* family and the genus *Parainfluenza*. It is a nonsegmented negative-stranded RNA virus, a murine correspondent of the human respiratory parainfluenza type 1 to 4 viruses, and a close cousin of mumps virus and measles virus, and it is more distantly related to human respiratory syncytial virus. The viral RNA of this family of viruses is more than 15,000 nucleotides long, complementary to the mRNA sense, and noninfectious. The minimum infectious unit is in fact the viral RNA wrapped in a helical nucleocapsid structure by about 2,600 copies of the NP protein and associated with 300 copies of the P/C and 40 copies of the L proteins (14), the latter two of which form the RNA polymerase complex (for a recent review, see reference 10). Because the basic rules governing encapsidation and replication are unclear, and also because of the large size of the viral RNA, it has not been possible up to now to produce infectious virus particles by transfecting cells with a plasmid expressing the viral RNA. Therefore, genetic manipulation of the viral genome is not yet possible for this category of viruses, consequently limiting the study of the viral functions and the development of other applications (e.g., vaccines).

In recent years, however, successful encapsidation and replication of shorter versions of the viral RNA synthesized from plasmids have been achieved by using viral functions expressed from cloned viral genes or provided by regular infections with the homologous virus (3, 4, 16, 19, 20). Two approaches have previously been taken for designing the shorter viral RNAs. On one hand, the RNA sequence has been constructed by flanking the reporter chloramphenicol acetyltransferase (CAT) gene sequence with the 3′ and 5′ viral RNA end sequences (4, 16, 19). On the other hand, the viral RNA was simply a copy of a defective interfering (DI) RNA naturally selected for its ability to efficiently replicate (3, 20). These two approaches obeyed different strategies. The former approach relied on the extreme sensitivity of the enzymatic assay performed by the reporter gene to detect replication. The latter approach relied on the ensured presence of all of the required sequences (some of which may have been omitted from the synthetic constructs) needed for efficient replication, thereby also ensuring that the reactions involved would be closer to those taking place in natural infections.

These systems, more than showing that proper encapsidation and replication of an RNA expressed from plasmid were feasible, opened the possibility of dissecting the viral functions as well as the *cis* RNA sequences involved. In this study, after we had observed that large internal deletions in the DI-H4 RNA obliterated its replication by the SV functions provided from plasmids, we constructed RNA derivatives with limited nucleotide modifications. From the replication pattern of the derivatives emerged the need for the total length of the RNA to be a multiple of 6 nucleotides. This conclusion is in agreement with the total number of nucleotides in the SV nondefective and DI-H4 RNAs and is consistent with the conclusions of previous structural studies of the nucleocapsid.

MATERIALS AND METHODS

Viruses and cells. SV Harris strain nondefective and DI-H4 copy-back defective viral stocks were prepared and characterized as described before (23). Vaccinia recombinant virus expressing the T7 RNA polymerase, vTF7-3, a gift from Bernard Moss (National Institutes of Health, Bethesda, Md.), has been described by Fuerst and colleagues (9) and was used accordingly. vTF7-3 stocks were prepared in Vero cells with titers ranging from 5×10^7 to 5×10^8 PFU/ml. CV1 and Vero cells were routinely grown in minimal Eagle medium supplemented with 5% fetal calf serum under a 5% CO_2 atmosphere.

Sequences and plasmids. The total SV RNA sequence is derived from the work of Shioda and colleagues (24, 25). It contains a total of 15,384 nucleotides. The pGem plasmids expressing the SV NP, P/C, and L genes under the control of the T7 RNA polymerase have been described previously (5, 12). The cloning and the description of the pSP65 plasmid containing the SV DI-H4 insert under the control of the T7 RNA polymerase promoter, pSV-DIH4/A, have been de-

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scribed by Calain et al. (3). In summary, this plasmid contains the sequence of the DI-H4 copy-back RNA (1,410 nucleotides long; see Results) under the control of the T7 RNA polymerase promoter. After linearization of the plasmid by *Bsm*I, the T7 RNA transcript represents an exact copy of the plus-strand DI-H4 RNA (3). Addition of the hepatitis delta virus genomic ribozyme (21, 22) to the end of the T7 RNA transcript was done in the following way. pSV-DIH4/A was digested with *Bsm*I, treated with Klenow to remove the 3′ protruding nucleotides, and finally digested with *Bam*HI flanking the *Bsm*I site to generate a blunted-*Bam*HI pSV-DIH4/A (3). The hepatitis delta virus genomic ribozyme sequence was recovered by polymerase chain reaction (PCR) amplification from plasmid HN 3-24 (27), a gift from Michael Lai (Los Angeles, Calif.), with primer Rib1 (5′ GGCCGGCATGGTCCC 3′) complementary to the ribozyme 5′ end and primer Rib3 (5′ CGAGGATCCGTC CCATTCGCCATTAC 3′) complementary to the 3′ ribozyme end sequence and flanked with a *Bam*HI site. The PCR product was digested with *Bam*HI and cloned into pSV-DIH4/A blunted-*Bam*HI (see above). The resulting plasmid was called pSV-DIH4/Rbz. Large deletions in the insert of pSV-DIH4/Rbz were generated by excisions of *Eco*RV (positions 958 and 1244), *Hpa*I-*Eco*RV (positions 461, 958, and 1244), or *Spe*I-*Eco*RV (positions 125, 153, 958, and 1244) fragment, followed by fill-in reactions, when adequate, and religations. The resulting plasmids were called, respectively, pSV-DIH4/RbzΔ1125, pSV-DIH4/RbzΔ628, and pSV-DIH4/RbzΔ296, with the last number referring to the resulting size of the DI-H4 insert (see Fig. 2). Limited stepwise modifications were created by opening pSV-DIH4/Rbz at unique sites (see Table 2). After treatment with 1 U of T4 DNA polymerase in standard buffer for 15 min at 12°C in the presence of 0.3 mM deoxynucleotide triphosphate (dNTP), the plasmids were religated. Every modification was verified by sequencing.

Ribozyme activity testing. To test the self-cleaving activity of the hepatitis delta virus ribozyme, the plasmids were linearized at the *Bam*HI site located 85 nucleotides downstream from the ribozyme cleavage site (these 85 nucleotides in fact represent the ribozyme sequence attached to the *Bam*HI site). The T7 RNA transcripts were then synthesized in the presence of [α-^{32}P]UTP in vitro with the T7 RNA polymerase (Biofinex, Praroman, Switzerland) according to the manufacturer's instructions. The labelled transcripts were then ethanol precipitated and separated on a 6% polyacrylamide gel to detect the 85-nucleotide fragment resulting from the self-cleavage activity. Negative controls for cleavage were made by cutting the pSV-DIH4/Rbz plasmids with *Bgl*I, which cleaves in the middle (38 nucleotides from the 5′ end) of the ribozyme sequence. The dried gels were quantitated in a phosphorimager (Molecular Dynamics) to estimate the percentage of cleavage.

Encapsidation-replication assays. For a complete replication assay, CV1 cells, seeded on 9-cm-diameter petri dishes, were infected with vTF7-3 at a multiplicity of infection of 2 to 3. One hour postinfection, the cells were transfected with pGem4-NP (5 μg), pGem4-P/C (5 μg), pGem4-L (1.5 μg), and pSV-DIH4/Rbz or its derivatives (5 μg) as previously described (3, 5). Cytoplasmic extracts were prepared 24 h postinfection, and CsCl gradient-purified nucleocapsid RNAs were analyzed by Northern (RNA) blotting (18). Replication was monitored by using a 5′ ex riboprobe (18) of positive polarity (same polarity as the T7-RNA transcript). Encapsidation was investigated in an assay from which pGem4-P/C and pGem4-L were omitted. The putative nucleocapsids were then purified and the nucleocapsid RNAs were analyzed by Northern blotting with a 5′ ex riboprobe of negative polarity (complementary to the T7 RNA transcripts [18]).

In vitro-synthesized T7 and SP6 RNA transcripts. In vitro RNA transcripts used as size and polarity markers were synthesized by T7 RNA or SP6 RNA polymerase (Biofinex), according to the supplier's instructions after proper linearization of pSV-DIH4 plasmids devoid of the ribozyme sequence (see Results).

Preparation of SV DI viral stocks from the DI nucleocapsids rescued from plasmids. The DI-H4 (or its derivative) nucleocapsids obtained in a replication assay were purified by banding on CsCl gradients, concentrated by centrifugation through glycerol onto a cushion of 68% sucrose in D_2O (SW55; 40,000 rpm, 12°C, 3 h), and transfected with 25 μl of TransfectACE (GIBCO BRL) into CV1 cells previously infected (2 h) with nondefective SV (multiplicity of infection = 20). After 20 h of infection, performed in the presence of 100 μg of cytosine arabinoside per ml to block contaminating vaccinia virus multiplication, the cells were recovered by 5 mM EDTA treatment and injected into 9-day-old embryonated chicken eggs (about 3×10^6 cells per egg). After 3 days of incubation at 33°C, the allantoic fluids (AFs) were collected and clarified by centrifugation (45 min, 12,000 × *g*). One milliliter of the AFs was mixed with 20 PFU of nondefective SV per cell and used to infect CV1 cells. The presence of infectious DI nucleocapsids originating from pSV-DI-H4 plasmids amplified in the CV1 cells was finally monitored by Northern blot and reverse PCR analysis of the intracellular viral nucleocapsid RNAs (see Fig. 5 for a schematic representation of the complete protocol).

PCR analysis. The viral nucleocapsids produced in the CV1 cells infected with the AFs obtained from the embryonated eggs (see above) were purified on a CsCl gradient. The nucleocapsid RNAs (isolated from about 5×10^6 cells) were then reverse transcribed with 20 pmol of the oligomer SV-LN6738 (15265-5′-TTCTGCACGATAGGGAC-3′-15248, with the numbers on each end referring to the position in the SV genome) of negative polarity with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 50-μl reaction mixtures according to the supplier's instructions. The cDNAs were diluted 1:10 in H_2O, and 5 μl was amplified with 20 pmol of oligomers SV-LN6738 and SV-B1 (14894-5′-GTTGAAGACAATTTC TAGAAGACT-3′-14917) as amplimers in a 100-μl reaction mixture containing 2.5 U of AmpliTaq DNA polymerase (Cetus)–3 mM Mg^{2+}–50 μM dNTP in a GeneAmp PCR System 9600 according to the supplier's instructions (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, 30 cycles). PCR amplifications of reverse transcriptase minus reactions were performed to control for contaminating plasmid DNA (see Results and Fig. 6).

RESULTS

Replication of DI-H4 RNA starting from plasmid pSV-DIH4/Rbz. We have previously shown that a T7 RNA transcript made from plasmid pSV-DIH4/A was successfully encapsidated and replicated by the SV functions NP, P, and L provided in *trans* by cotransfection with pGem plasmids (3). In those experiments, the correct 3′ extremity of the RNA transcript was generated by *Bsm*I linearization of pSV-DIH4/A before transfection. This method of transfection is generally viewed as a low-efficiency transfection method, and accordingly, this was reflected in the poor

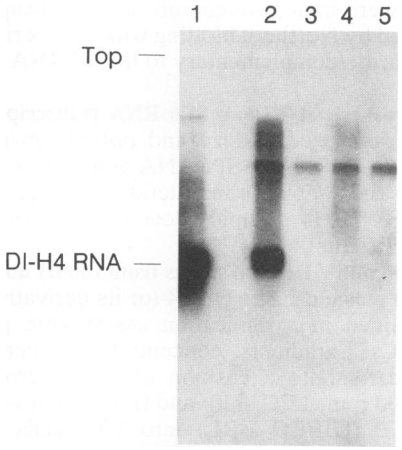

FIG. 1. Replication of DI-H4 RNA from plasmid pSV-DIH4/Rbz. About 10 million CV1 cells were infected with vTF7-3 and transfected with pSV-DIH4/Rbz (lanes 2 and 3) or pSV-DIH4/A (lanes 4 and 5) either with pGem-NP, -P/C, and -L (lanes 2 and 4) or without these viral replicating functions (lanes 3 and 5). Twenty-four hours posttransfection, the nucleocapsids were purified and the viral RNA was analyzed by Northern blotting as described in Materials and Methods. The 5′ ex riboprobe used is of positive polarity. In lane 1, 1/200th of a similar amount of CV1 cells infected with the SV viral stock DI-H4, in the presence of vTF7-3, is similarly analyzed. The molecular weight marker indicating the size of the natural DI-H4 RNA is shown (lane 1).

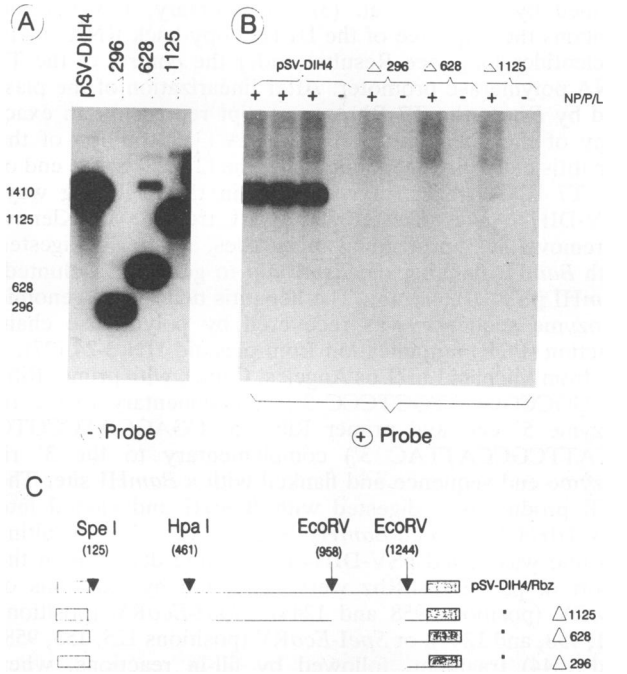

FIG. 2. Unsuccessful replication of DI-H4 RNAs with large internal deletions. (A) In vitro T7 RNA transcripts made from plasmids pSV-DIH4/A, pSV-DIH4-Δ1125, pSV-DIH4-Δ628, and pSV-DIH4-Δ296. (B) Nucleocapsid-purified RNAs isolated from replication assays after transfections with pSV-DIH4/Rbz, pSV-DIH4/Rbz-Δ296, pSV-DIH4/Rbz-Δ628, and pSV-DIH4/Rbz-Δ1125, with or without the viral replicating functions. Transfections with pSV-DIH4/Rbz are presented in triplicate. In panels A and B, the RNAs are analyzed in identical Northern blots and the 5′ ex riboprobes are of negative and positive polarities, respectively. (C) Schematic description of the deletions. Shaded boxes represent inverted repeats of the DI RNAs. The numbers in the designations of the deleted plasmids refer to the final size of the DI RNA sequence.

reproducibility of the reaction as well as in the efficiency of replication, which was often at the limit of Northern blot detection (3). To avoid such a drawback, the self-cleaving ribozyme sequence of the hepatitis delta virus was cloned into the pSV-DIH4/A to generate plasmid pSV-DIH4/Rbz. Replication assays using pSV-DIH4/Rbz to provide the DI-H4 RNA were indeed found to be more consistent and efficient. A typical example is presented in Fig. 1, in which the results of assays using pSV-DIH4/Rbz or pSV-DIH4/A are shown side by side. Under conditions in which transfection with pSV-DIH4/Rbz allowed the detection of a strong replication signal (lane 2), the use of *Bsm*I-linearized pSV-DIH4/A (lane 4) showed no signal above the background (lane 5) at this level of gel exposure. Although the efficiency of replication varied by factors of 2 to 5 from experiment to experiment, in more than 15 different experiments no failure to replicate DI-H4 RNA from pSV-DIH4/Rbz was ever observed. In these experiments, the criteria for assessing replication are identical to those used earlier (3), i.e., (i) the RNA was purified in the form of a nucleocapsid banding at the right density in a CsCl gradient and is resistant to ribonuclease digestion (not shown), (ii) it is complementary to the T7 RNA transcript (the riboprobe used in the Northern blot is of the same polarity as this transcript), and (iii) its presence depended on the cotransfection of all three SV replicating functions (Fig. 1, lane 3).

Large internal deletions in DI-H4 sequence obliterate replication. Confident that the replication assay was very reproducible, we made large internal deletions in the DI-H4 sequence in an attempt to estimate the effect of total RNA size on the efficiency of replication. As shown in Fig. 2C, the internal deletions left untouched the ends of the RNAs; even the inverted repeats of 110 nucleotides were conserved. Surprisingly, none of the deleted RNAs was replicated in conditions in which integral DI-H4 RNA was replicated (Fig. 2B). As shown in Fig. 2A, the deleted plasmids were as competent as pSV-DIH4 for synthesizing the T7 transcripts. Moreover, no reduction of the self-cleaving activity used to generate the exact 3′ end of the transcript could be observed (Table 1).

Effects of limited internal deletions in DI-H4 RNA on replication. Explanations to account for the failure in replication reported above could be, among others, the removal

TABLE 1. Self-cleaving activities of large DI-H4 RNA deletions[a]

Derivative of pSV-DIH4/Rbz	Arbitrary U of radioactive signal in:		Size of 85-mer (%)[b]	Amt of radioactivity in 85-mer (%)[c]	% Cleavage[d]
	Top bands	85-mer			
	633,970	9,586	5.40	1.50	28
-1125	1,020,019	20,318	6.60	2.00	30
-628	560,712	18,759	11.30	3.30	29
-296	123,370	12,982	21.40	10.50	49

[a] For details on the procedure used to estimate self-cleaving activity, see Materials and Methods.
[b] Relative to that of the total transcript.
[c] Relative to that in the total transcript.
[d] Determined by dividing amount of radioactivity in 85-mer by size of 85-mer.

TABLE 2. Replication ability of DI-H4 RNA and its derivatives[a]

Plasmid or derivative	Sites (429) Cel II	(980) BstX1	(1026) Mun I	(1068) Sau I	Sau I*	(1239) Dra III	Total base modification	Replication	Encapsidation	Position in six phase
Nb. of bases changed	+3	-4	+4	+3	+2	-3				
pSV-DIH4/Rbz	−	−	−	−	−	−	None	+	+	0
pSV-DIH4/Rbz										
-01	+	−	−	−	−	−	+3	−	ND[b]	+3
-02	−	+	−	−	−	−	−4	−	ND	+2
-03	−	−	+	−	−	−	+4	−	ND	+4
-04	−	−	−	−	+	−	+2	−	ND	+2
-05	−	−	−	−	−	+	−3	−	ND	+3
-06	+	+	−	−	−	−	−1	−	ND	+5
-07	+	−	+	−	−	−	+7	−	ND	+1
-08	+	−	−	−	+	−	+5	−	ND	+5
-09	−	+	−	+	−	−	−1	−	ND	+5
-10	−	+	−		−	+	−7	−	ND	+5
-11	−	−	+	+	−	−	+7	−	+	+1
-12	−	−	+	−	−	+	+1	−	+	+1
-13	+	−	−	−	−	+	0	+	ND	0
-14	−	+	+	−	−	−	0	+	ND	0
-15	−	−	−	+	−	+	0	+	ND	0
-16	+	−	−	+	−	−	+6	+	ND	0
-17	−	−	+	−	+	−	+6	+	+	0

[a] Sau I*, variant of *Sau*I site modification that yielded only a 2-base addition.
[b] ND, not done.

of *cis*-acting essential sequences, the generation of DI-H4 RNAs too short to support replication, or some structural requirements of the nucleocapsids preventing any change in the number of nucleotides. These possibilities were made likely by the large size deletions performed. Care was then taken to disrupt as little as possible the RNA sequence in order to learn the rules governing replication. Consequently, 2- to 7-nucleotide deletions or insertions only were produced by cutting and filling in at different unique sites along the sequence. A total of 17 derivatives of pSV-DIH4/Rbz were produced in this way (Table 2, pSV-DIH4/Rbz-01 through -17) and checked in replication assays. Figure 3 presents the Northern blot analysis of the five derivatives (samples 14, 15, 13, 16, and 17) which were successfully and efficiently amplified, and of a selected sample of four derivatives (samples 02, 03, 05, and 01) which were, relative to the previous samples, negative for replication, although a faint signal could sometimes be detected (see in this particular experiment samples 03 and 01). Table 2 summarizes all of the results obtained. The following observations are relevant. (i) The addition of a total of 1, 2, 3, 4, 5, or 7 nucleotides led to negative results, as did the removal of a total of 1, 3, 4, or 7 nucleotides, regardless of the site of modification (Table 2, pSV-DIH4/Rbz and pSV-DIH4/Rbz-01 through -12). (ii) The addition of 3 or 4 nucleotides, with the compensatory removal of 3 or 4 nucleotides at different sites, reestablished the high replication ability (pSV-DIH4/Rbz-13 through -15). (iii) The addition of a total of 6 nucleotides by stepwise additions of 3 plus 3 or 4 plus 2 nucleotides at different locations led to efficient replication (pSV-DIH4/Rbz-16 and -17). (iv) The resulting number of nucleotide changes and not the position of the changes appeared to be important. For instance, a 3-base addition at the *Cel*II site abolished replication (sample pSV-DIH4/Rbz-01). The same modification, when corrected by a 3-base removal at the *Dra*III site (sample pSV-DIH4/Rbz-13) or by a 3-base addition at the *Sau*I site (sample pSV-DIH4/Rbz-16), was no longer negative. As in the case of large internal deletions, the negative results seen here could not be explained by a lack of T7 RNA transcript synthesis nor by a lack of ribozyme activity (not shown).

Encapsidation of nonreplicated DI-H4 RNAs. For replication to take place, a proper template recognized by the viral polymerase has to first be formed. This step involves encapsidation by the NP protein of the T7 RNA transcripts. Encapsidation must, of course, have taken place for the DI RNAs to have successfully replicated. Whether this was the case for the derivatives that did not replicate was open to question. Two replicating and two nonreplicating DI-H4 RNAs were therefore compared for their abilities to be encapsidated when synthesized in the presence of NP alone (Fig. 4). Figure 4A illustrates the replication abilities of the RNAs used. In the presence of the three SV functions but not in the presence of NP alone, DI-H4 RNA and the derivative pSV-DIH4/Rbz-17 efficiently replicated, while derivatives pSV-DIH4/Rbz-12 and -11 did not. However, examination of the T7 RNA transcripts (with a probe of negative polarity [Fig. 4B]) showed that in the presence of NP alone (or in conjunction with P/C and L) these transcripts were all found in nucleocapsid structures (banding in CsCl gradients). Note that the RNAs recovered from the nucleocapsids in the absence of replication were slightly smaller than those seen in the presence of replication (for the most obvious example, compare lanes 3 and 12 with lanes 5 and 14 in Fig. 4B). This is likely to reflect the presence on the agarose gel of only the plus-sense transcript in the absence of

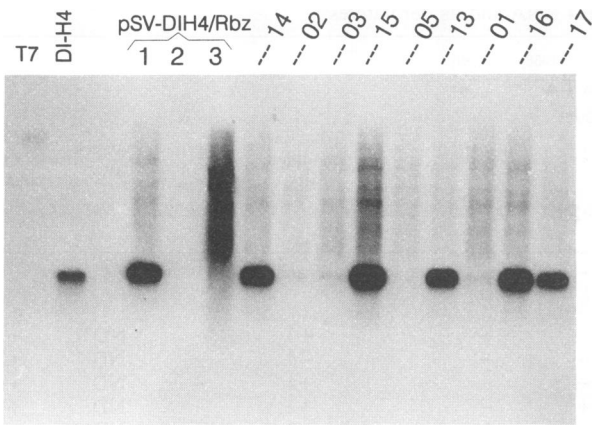

FIG. 3. Replication of DI-H4 RNAs with limited internal modifications. Northern blot analysis of nucleocapsid-purified RNAs from cells transfected with the plasmids expressing the DI-H4 RNA and their derivatives (for a description, see Table 2). For all of the rescue experiments (lanes 1 and 14 through 17), pGem-NP, -P/C, and -L were cotransfected with the plasmid expressing the DI-H4 RNA, except for two samples using pSV-DIH4/Rbz from which the three functions were omitted (lane 2) or in which only pGem-NP was used (lane 3). In vitro T7 RNA transcript synthesized from pSV-DIH4/A linearized with *Bam*HI (lane T7) and nucleocapsid RNA isolated from cells infected with SV DI-H4 (lane DI-H4) are also shown. A 5′ ex riboprobe of positive polarity has been used.

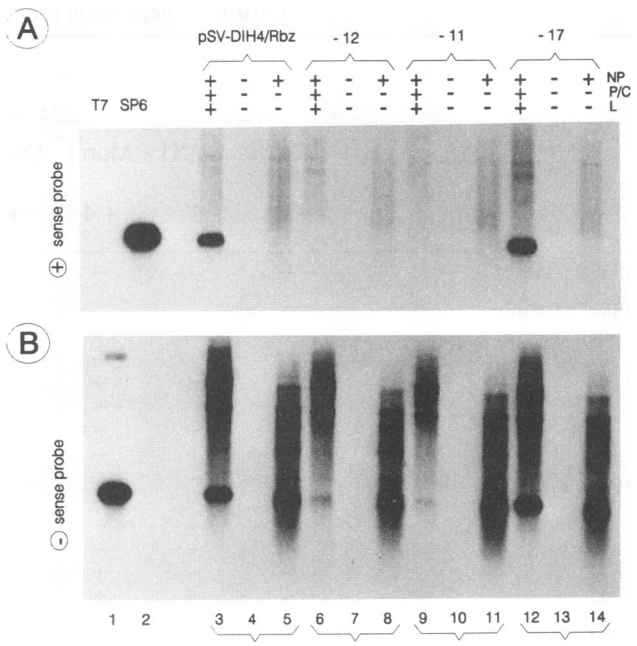

FIG. 4. Encapsidation of DI-H4 RNAs and their derivatives. pSV-DIH4/Rbz and its derivatives pSV-DIH4/Rbz-12, -11, and -17 (see Table 2 for a description) were transfected into CV1 cells, with or without the plasmids expressing the SV replicating functions (NP, P/C, and L) as indicated, in replication or encapsidation assays (see Materials and Methods). Viral nucleocapsids were isolated and the presence of the DI-H4 RNAs was monitored by Northern blot analysis. Lanes: T7 and SP6, in vitro RNA transcripts synthesized from pSV-DIH4/A with T7 and SP6 RNA polymerases, respectively. 5′ ex riboprobes of negative or positive polarity have been used as indicated.

replication, in contrast to the presence of the plus- and minus-sense RNAs when replication takes place. Also note in Fig. 4B the higher-molecular-weight molecules reacting with the minus-strand probe. Most of these molecules were found to be resistant to ribonuclease digestion (data not shown). They are likely to represent mostly encapsidated large T7 RNA transcripts, since they were purified as nucleocapsids. They obviously did not replicate, as evidenced by the absence of their negative-strand counterparts in Fig. 4A, in which residual plasmid contamination is probably responsible for the faint signals observed above the DI RNA bands (Fig. 1, lanes 1 to 5).

DI-H4 RNA replicated from plasmid is infectious. To verify that the nucleocapsids containing DI-H4 RNAs originating from plasmids represented fully competent viral nucleocapsids, a method to produce competent viral stocks containing these nucleocapsids was developed (presented in Fig. 5A). The multiple steps involved were required to provide the nucleocapsids with a viral envelope (adding nondefective virus in step 2) and to produce infectious virus (passage into embryonated eggs in step 3). In step 5, nondefective virus was added to ensure an efficient amplication of the putative infectious DI virus produced in eggs. According to this scheme, DI-H4 RNAs should only be amplified in step 5 when efficiently replicated in step 1. A control in this experiment was, therefore, to omit the three replicating functions in step 1. Figure 5B shows that indeed DI-H4 RNAs were amplified in step 5 (samples $H4^+$ and 17^+). In contrast, they were not detected when replication was not made possible in step 1 (samples $H4^-$ and 17^-). This demonstrated that the AFs contained infectious viral particles with DI-H4 RNAs originating from plasmids. In these experiments, two types of DI-H4 RNAs were used: (i) the original DI-H4 RNA, synthesized from pSV-DIH4/Rbz, whose sequence aligns with that of the nondefective RNA, except for the inverted repeats, and (ii) that produced by pSV-DIH4/Rbz-17, in which 4 and 2 nucleotides were added, at the *Mun*I and *Sau*I sites, respectively, thereby destroying these sites (Table 2). To confirm that the DI RNAs that replicated in step 5 originated from plasmids, a region covering the two *Mun*I and *Sau*I sites was amplified by reverse PCR (see Fig. 6A). The PCR products were then assayed for sensitivity to *Mun*I and *Sau*I digestions. Figure 6B shows the successful amplification of the expected DNA fragment from the four samples presented in Fig. 5B. Not surprising was the recovery of the PCR product from samples $H4^-$ and 17^-, in which DI-H4 did not replicate, since nondefective RNA could also serve as template for the reaction. Note, however, that these PCR products could not be found in the absence of the reverse transcription reaction (RTase − lanes in Fig. 6B), excluding the fact that the plasmids used in the original transfections (step 1 in Fig. 5A) could have been carried along. Digestions with *Mun*I and *Sau*I are presented in Fig. 6C. In the control samples, the 371-nucleotide PCR products originated from pSV-DIH4/Rbz-17 showed total resistance (sample p17), in contrast to sample pH4, which was made from plasmid pSV-DIH4/Rbz. Of the four samples produced from the RNAs presented in Fig. 5 (samples $H4^+$, $H4^-$, 17^+, and 17^-), only the one containing DI-RNA-17 (17^+) led to a PCR product resistant to both restriction enzymes. All of the samples contained, on the other hand, a 371-base fragment sensitive to digestion, since they all contained nondefective viral RNA which served equally as a template for the reverse PCRs. This experiment unequivocally demonstrated that the RNAs orig-

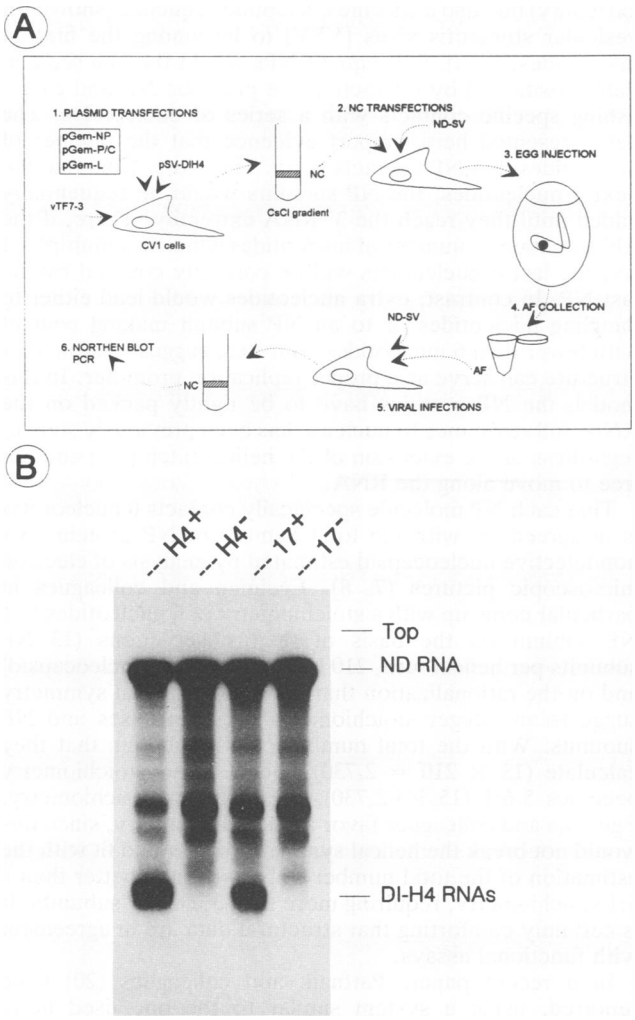

FIG. 5. DI-H4 RNAs produced from plasmids are enveloped into competent virus particles. (A) Scheme of the steps leading to infectious virus from plasmid transfection (for more details, see Materials and Methods). In step 2, the nucleocapsids (NC) isolated from step 1 were transfected and infected with nondefective (ND) SV. In step 3, the whole cells were injected into the embryonated eggs. In step 5, AF collected from the eggs was mixed with nondefective SV. In step 6, the PCR analysis presented in Fig. 6 is shown. (B) Northern blot analysis of the nucleocapsid RNAs amplified in step 5. H4 or 17 samples in their respective lanes are nucleocapsid RNAs isolated from assays with, in the original transfection (step 1), respectively, pSV-DIH4/Rbz and pSV-DIH4/Rbz-17, with or without the three replicating functions (samples $H4^+$ and 17^+ and $H4^-$ and 17^-). The whole procedure was carried out in parallel for each of the four samples. ND RNA, nondefective RNA.

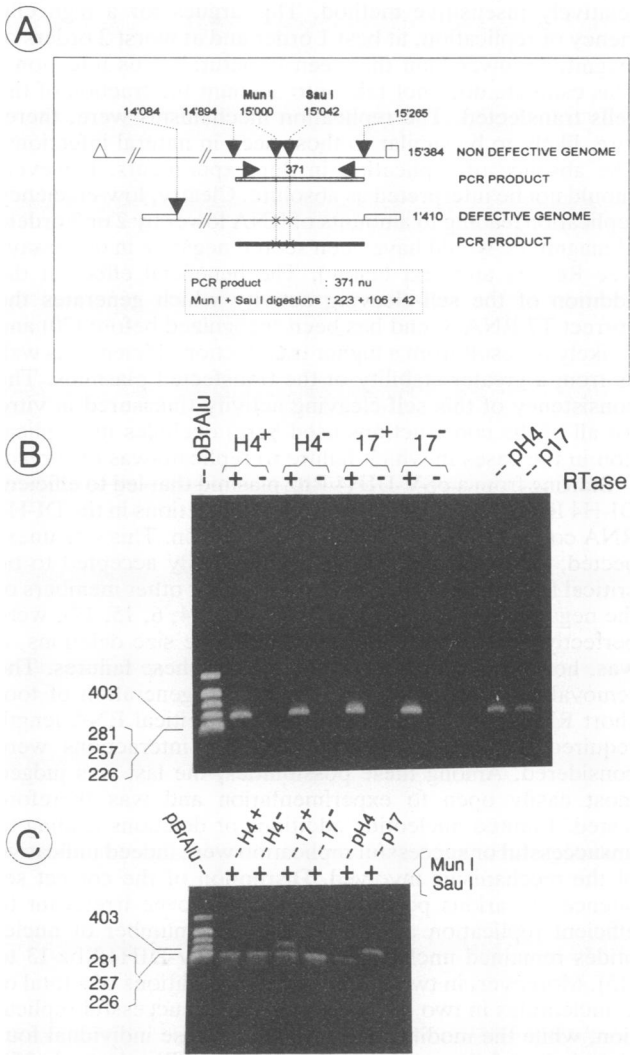

FIG. 6. Reverse PCR analysis of the DI-H4 RNAs originating from plasmids and properly enveloped in competent virus particles. (A) Scheme of the reverse PCR method (for details about primers and reactions, see Materials and Methods). The reverse PCR amplifies a product of 371 nucleotides from both the nondefective and the DI SV RNAs. When amplified from pSV-DIH4/Rbz-17, however, this product is resistant to digestion with *Mun*I and *Sau*I. (B) A 1% agarose gel analysis of the reverse PCR products obtained with nucleocapsid RNAs purified from cells in step 5 as templates (Fig. 5A) after the original transfection (step 1 in Fig. 5A) with either pSV-DIH4/Rbz (samples H4) or pSV-DIH4/Rbz-17 (samples 17) with or without the three replicating functions (samples $H4^+$ and 17^+ and $H4^-$ and 17^-). Lanes pH4 and p17 contain PCR products of reactions with pSV-DIH4/Rbz and pSV-DIH4/Rbz-17 plasmids. Row RTase indicates whether reverse PCRs included the reverse transcriptase. Molecular markers (in bases) are on the left. (C) A 1% agarose gel analysis of the sensitivity of the PCR products to digestion with *Mun*I or *Sau*I. For details, see the legend to panel B.

inating from plasmids were not only efficiently replicated but behaved like bona fide subgenomic viral RNAs in that they were replicated by the helper virus and enveloped in competent virus particles.

Total length of the DI-H4 insert in pSV-DIH4/Rbz. When DI-H4 RNA was cloned into pSV-DIH4/A, an estimate of its length based on partial sequencing of crucial regions of the insert and on alignment with published sequences (24, 25) led to a total of 1,411 nucleotides (3). Since then, the complete sequence of the insert has been done (data not shown). A single-base deletion was found at position 111 downstream from the genomic 3′ end (for details, see reference 3). Therefore, the total number of nucleotides in the insert is 1,410.

DISCUSSION

In the experiments presented here, the replication of DI RNAs from plasmids was detected by Northern blotting, a

relatively insensitive method. This argues for a high efficiency of replication, at best 1 order and at worst 2 orders of magnitude lower than that seen in natural virus infections. This estimate does not take into account the fraction of the cells transfected. The replication mechanisms were, therefore, likely to be similar to those seen in natural infections. The absence of replication in our experiments, however, should not be interpreted as absolute. Clearly, low-efficiency replication leading to amounts of RNA lower by 2 or 3 orders of magnitude would have been scored negative in our assays (see Results and text below). The beneficial effect of the addition of the self-cleaving activity which generates the correct T7 RNA 3′ end has been recognized before (20) and is likely to result from a higher transfection efficiency as well as from a greater stability of the transfected plasmids. The consistency of this self-cleaving activity (measured in vitro for all of the constructions used here) excludes its implication in the cases in which failure to replicate was observed.

Starting from a pSV-DIH4/Rbz plasmid that led to efficient DI-H4 RNA replication, large internal deletions in the DI-H4 RNA coding sequence abolished replication. This was unexpected, since the end sequences, generally accepted to be critical for replication for SV as well as for other members of the negative-stranded RNA virus family (4, 6, 15, 17), were perfectly conserved. Because of the large size deletions, it was, however, difficult to easily explain these failures. The removal of essential *cis* sequences, the generation of too-short RNAs, and the modification of a critical RNA length required to provide specific NP-RNA interactions were considered. Among these possibilities, the last was judged most easily open to experimentation and was therefore tested. Limited nucleotide additions or deletions leading to unsuccessful or successful replication were indeed indicative of the mechanisms involved. Disruption of the correct sequence at various positions was found to be irrelevant to efficient replication as long as the total number of nucleotides remained unchanged (Table 2, pSV-DIH4/Rbz-13 to -15). Moreover, in two cases, two-step additions of a total of 6 nucleotides in two different sites led to successful replication, while the modification of each of these individual four sites led to failures (Table 2, pSV-DIH4/Rbz-16 and -17). Therefore, if the total number of nucleotides appeared critical, the addition of 6 nucleotides was allowed. The simplest interpretation of these results is that the total number of nucleotides must be a multiple of six. This rule (rule of six) is verified in our experiments (Table 2, last column) and is also in agreement with the total number of nucleotides of the nondefective genome and of the DI-H4 genome produced from pSV-DIH4/Rbz, which amount to 15,384 (24, 25) and 1,410, respectively. This rule provides a likely explanation for the failure to replicate the large internal deletions as well, since the DI-H4 RNAs were shortened by 285, 782, and 1,114 nucleotides; none of these numbers is a multiple of six. Other causes for failure are possible.

In the failures to replicate, encapsidation of the T7 transcripts (starting at the 5′ end) did not appear to be the limiting step, since the transcripts were found in nucleocapsid structures banding on CsCl gradients (Fig. 4). Assuming, therefore, that correct encapsidation of the T7 transcripts takes place anyway, the critical step for allowing replication may become the correct encapsidation of the 3′ extremity. This extremity is crucial in that it represents the promoter for the synthesis of the strand complementary to the T7 RNA transcript. The 5′ end contains the signal for proper positioning of the first NP subunit and for encapsidation initiation this signal is likely to be not only geographic (the 5′ extremity) but also a specific nucleotide sequence (shown for vesicular stomatitis virus [VSV] to lie among the first 14 nucleotides [1, 2]). Subsequent NPs would then be sequentially positioned by contacting the previous NP and establishing specific contacts with a series of nucleotides. The data presented here support evidence that the number of nucleotides that NP contacts is six. Therefore, from 6 to the next 6 nucleotides, the NP subunits would be sequentially added until they reach the 3′ RNA extremity. There, if the RNA contains a number of nucleotides which is a multiple of six, the last 6 nucleotides will be correctly covered by the last NP. In contrast, extra nucleotides would lead either to dangling nucleotides or to an NP subunit making contact with fewer than 6 nucleotides. Our data suggest that neither structure can serve as a proper replication promoter. In this model, the NP proteins have to be tightly packed on the RNA (full resistance to nuclease has been previously shown, regardless of the extension of the helical pitch [11]) and not free to move along the RNA.

That each NP molecule specifically contacts 6 nucleotides is in agreement with the total number of NP proteins per nondefective nucleocapsid estimated by analysis of electron microscopic pictures (7, 8). Egelman and colleagues in particular come up with a stoichiometry of 6 nucleotides to 1 NP subunit on the basis of their observations (13 NP subunits per helical turn, 210 helical turns per nucleocapsid) and on the rationalization that nonbroken helical symmetry suggests an integer stoichiometry between bases and NP subunits. With the total number of NP subunits that they calculate ($13 \times 210 = 2{,}730$), the deduced stoichiometry becomes 5.6:1 (15,384/2,730), a noninteger stoichiometry. Egelman and colleagues favor a 6:1 stoichiometry, since this would not break the helical symmetry and would fit with the estimation of the total number of NP subunits better than a 5:1 stoichiometry, requiring more than 3,000 NP subunits. It is certainly comforting that structural data are in agreement with functional assays.

In a recent paper, Pattnaik and colleagues (20) have reported, using a system similar to the one used here, efficient encapsidation and replication of a VSV DI RNA expressed from plasmid. They made the interesting observation that 4 to 5 extra nucleotides at the 5′-end T7 transcript were tolerated, in that they did not kill replication. They were, however, trimmed in the DI RNA that was successfully amplified. On the other hand, as few as 2 extra nucleotides at the 3′ end of the T7 transcript abolished replication. In view of the results presented here, and assuming that a basic rule such as the rule of six applies to VSV, these results can be interpreted as follows. Correct positioning of the first NP only takes place internally at the true viral RNA 5′ end by recognition of a specific nucleotide sequence (leaving dangling extra nucleotides). Encapsidation would then proceed to the 3′ end, regardless of the nucleotide sequence, but with the correct integer NP/nucleotide stoichiometry observed. At the 3′ end, only the precise number of nucleotides would be tolerated to generate the replication promoter for the complementary strand (no extra nucleotides allowed). That the synthesis of the complementary strand leads to a successful template for the next round of replication, despite the dangling 5′ extra nucleotides, could be explained if the unprotected 5′ nucleotides are cleaved. Alternatively, they could not be copied by the viral replicase, because they are not covered by NP. That correction at the 3′ end does not take place (no trimming) may suggest that extra nucleotides are not dangling but rather are covered with an extra NP protein, enough to be protected,

but not properly so that a correct replication promoter is formed.

The rule of six does not preclude the need for a particular nucleotide sequence at the RNA ends. As suggested before and mentioned above, these 11 to 14 nucleotides conserved between the plus and minus strands are likely to constitute, when in the proper phase with the NP proteins, a specific signal required for forming an adequate template for replication. Moreover, the rule of six does not exclude other possible requirements, such as total minimal RNA length or particular *cis* RNA sequences. Note that an RNA construct containing the CAT gene sequence flanked by the SV 3′- and 5′-end sequences has been successfully replicated with nondefective SV to provide the helper functions (19). The total number of nucleotides in this RNA amounts to 924, a multiple of six. However, the replication of this RNA lacking all but the 145 nucleotides at the 3′-end and 119 nucleotides at the 5′-end SV sequences was only detected by measurement of the CAT activity (a highly sensitive assay compared with Northern blotting). This could suggest the need for internal sequences, absent from this synthetic construct, to reach higher replication efficiency. Alternatively, this could reflect the difference in plus- and minus-strand replication promoters. In copy-back DI RNAs, the promoter responsible for minus-strand synthesis (generally accepted to be of high efficiency) is present on both RNA strands (contained in the inverted repeat). This is obviously not the case for the RNA synthetic construct (analogous to an internal deletion DI RNA), which has retained the ends of the genomic RNA. Note that when a synthetic RNA construct of the type reported by Park et al. (19) was confronted with the rule of six, the correct number of nucleotides had a positive effect (three- to fivefold) on the efficiency of replication. This, however, was still marginal (i.e., only detected after PCR amplification) compared with the level of replication reached with the natural copy-back DI RNA (11a).

It is therefore possible that the rule of six shows a significant effect on replication only when all of the other rules needed for replication are observed. The rule of six would thus become necessary for efficient replication in conjunction with other rules needed for replication. This interpretation could account for the out-of-phase derivatives exhibiting sporadic replication abilities at the limit of detection (Fig. 3, samples 03 and 01) or for a marginal effect on synthetic constructs obeying the rule of six (cf. constructs in reference 11a).

Successful replication by negative-stranded RNA virus or virus functions of RNAs produced from plasmids has been reported for other viruses as well. Apart from the VSV DI RNA and the SV CAT construct already discussed above, RNA molecules containing the reporter gene CAT sequence flanked by 3′ and 5′ viral RNA end sequences of influenza A or respiratory syncytial viruses have been replicated by their respective homologous viruses (4, 16). These artificial defective RNAs were correctly enveloped in virus particles. Among these different RNAs, only the VSV DI RNA replicated with an efficiency comparable to that observed here (direct detection of the DI RNA), therefore potentially fulfilling the conditions for the rule of six to apply. However, although the natural VSV DI RNA is 2,208 (a multiple of six) nucleotides long, the DI RNA which was reported to efficiently replicate from plasmid is 1 nucleotide longer (20). Moreover, Wertz and colleagues recently reported the successful replication of a series of deletions of their original DI RNA for which a strict correlation with the rule of six was not observed (26). Among the CAT constructs, for which the rule of six may not apply with full potential according to our interpretation (see above), the influenza constructs of Luytjes et al. (16) fitted the rule. This, however, has to be balanced by the fact that for influenza virus, none of the natural full-length genes are made up of nucleotides in multiples of six (see reference 13 for a review).

In conclusion, the available sequence data do not, at present, speak for a wide application of the rule of six. It is not clear whether a more extended sequence comparison will bring more information, since for most of the reported sequences, the degree of confidence does not lie within plus or minus 1 or 2 nucleotides. Rather than elucubrate the general prevalence of the rule of six, one would like to propose that although the number of nucleotides per nucleocapsid protein unit may depend on the type of virus (on the size of the nucleocapsid protein), the rule that the stoichiometry of nucleotide per NP protein must be an integer has to be considered for all of the viruses containing a nucleocapsid with tight helical symmetry.

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REFERENCES

1. **Blumberg, B. M., C. Giorgi, and D. Kolakofsky.** 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. Cell **32**:559–567.
2. **Blumberg, B. M., and D. Kolakofsky.** 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. J. Virol. **40**:568–576.
3. **Calain, P., J. Curran, D. Kolakofsky, and L. Roux.** 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology **191**:62–71.
4. **Collins, P. L., M. A. Mink, and D. S. Stec.** 1991. Rescue of synthetic analogs of respiratory syncytial genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc. Natl. Acad. Sci. USA **88**:9663–9667.
5. **Curran, J., R. Boeck, and D. Kolakofsky.** 1991. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. **10**:3079–3085.
6. **Curran, J. A., and D. Kolakofsky.** 1991. Rescue of a Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. Virology **182**:168–176.
7. **Egelman, E. H., S.-S. Wu, M. Amrein, A. Portner, and G. Murti.** 1989. The Sendai virus nucleocapsid exists in at least four different helical states. J. Virol. **63**:2233–2243.
8. **Finch, J. T., and A. J. Gibbs.** 1970. Observations on the structure of the nucleocapsids of some paramyxoviruses. J. Gen. Virol. **6**:141–150.
9. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
10. **Galinsky, M. S., and S. L. Wechsler.** 1991. The molecular biology of the paramyxovirus genus, p. 41–82. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
11. **Heggeness, M. H., A. Scheid, and P. W. Choppin.** 1981. The relationship of conformational changes in the Sendai virus

nucleocapsid to proteolytic cleavage of the NP polypeptide. Virology **114**:555–562.

11a. **Jacques, J.-P., and D. Kolakofsky.** Personal communication.

12. **Kast, W. M., L. Roux, J. Curran, H. J. J. Blom, A. C. Voordouw, R. H. Meloen, D. Kolakofsky, and C. J. M. Melief.** 1991. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. Proc. Natl. Acad. Sci. USA **88**:2283–2287.

13. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. **52**:467–506.

14. **Lamb, R. A., B. W. J. Mahy, and P. W. Choppin.** 1976. The synthesis of Sendai virus polypeptides in infected cells. Virology **69**:116–131.

15. **Li, X. Q., and P. Palese.** 1992. Mutational analysis of the promoter required for influenza virus virion RNA synthesis. J. Virol. **66**:4331–4338.

16. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell **59**:1107–1113.

17. **Mink, M. A., D. S. Stec, and P. L. Collins.** 1991. Nucleotide sequences of the 3′ leader and 5′ trailer regions of human respiratory syncytial virus genomic RNA. Virology **185**:615–624.

18. **Mottet, G., and L. Roux.** 1989. Budding efficiency of Sendai virus nucleocapsids: influence of size and ends of the RNA. Virus Res. **14**:175–188.

19. **Park, K. H., T. Huang, F. F. Correia, and M. Krystal.** 1991. Rescue of a foreign gene by Sendai virus. Proc. Natl. Acad. Sci. USA **88**:5537–5541.

20. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69**:1011–1020.

21. **Perrotta, A. T., and M. D. Been.** 1990. The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturants. Nucleic Acids Res. **18**:6821–6827.

22. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) **350**:434–436.

23. **Roux, L., and J. J. Holland.** 1979. Role of defective interfering particles of Sendai virus in persistent infections. Virology **93**:91–103.

24. **Shioda, T., Y. Hidaka, T. Kanda, H. Shibuta, A. Nomoto, and K. Iwasaki.** 1983. Sequence of 3,687 nucleotides from the 3′ end of Sendai virus genome RNA and the predicted amino acid sequences of viral NP, P and C proteins. Nucleic Acids Res. **11**:7317–7330.

25. **Shioda, T., K. Iwasaki, and H. Shibuta.** 1986. Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN, and L proteins. Nucleic Acids Res. **14**:1545–1563.

26. **Wertz, G. W. et al.** 1993. Presented at the Reverse Genetics of Negative-Stranded RNA Viruses Meeting, Madrid, February 1993.

27. **Wu, H.-N., Y.-J. Lin, F.-P. Lin, S. Makino, M.-F. Chang, and M. M. C. Lai.** 1989. Human hepatitis delta virus RNA subfragments contain an autocleavable activity. Proc. Natl. Acad. Sci. USA **86**:1831–1835.