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Genome Nucleotide Lengths That Are Divisible by Six Are Not Essential but Enhance Replication of Defective Interfering RNAs of the Paramyxovirus Simian Virus 5

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For some members of the Paramyxoviridae family of negative strand RNA viruses, efficient genome replication only occurs when the total genome length is a multiple of six (6*N* length, where *N* is any integer). To determine if this ''rule of six'' requirement applied to the replication of the prototype paramyxovirus simian virus 5 (SV5), defective interfering (DI) RNA genomes were generated by sequential undiluted passage of virus in tissue culture. Molecular cloning and nucleotide sequence analysis of 10 RNA genomes revealed a series of copyback DI RNAs with chain lengths between 449 and 1365 bases, but only 4 of the 10 naturally occurring RNA genomes were of 6*N* length. Many of the cloned DI genomes could be grouped into two distinct nested sets, with the members of each set having the same polymerase crossover junctions and extent of terminal complementarity but differing from each other by internal deletions. One of these nested sets of genomes consisted of novel DI RNAs that contained a pentameric stretch of nontemplated adenosine residues inserted precisely at the polymerase crossover junction. A reverse genetics system was established in which SV5 DI genomes were replicated *in vivo* entirely by cDNA-derived components. Using this system, two naturally occurring SV5 DI RNAs were examined in a mutational analysis to determine the role of genome length on SV5 RNA replication. The progressive insertion of one to six nucleotides into a 6*N* length DI genome (852 bases) resulted in a reduction in replication for RNAs that contained one to four additional bases (~35–50% of WT levels), followed by an increase back to WT replication levels for genomes that were altered by five and six base insertions (~70 and 100% of WT levels, respectively). An insertion of five nucleotides into a second non-6*N* length DI RNA (499 total bases) created a genome length that was a multiple of six (504 bases) and led to a ~10-fold stimulation of replication over that of the unaltered genome. Together, these results indicate that there was a clear influence of 6*N* genome length on SV5 DI RNA replication, but the stringency of this replication requirement appeared to be less than that found previously for other paramyxoviruses. This work completes the testing of the rule of six replication requirement for representatives of each of the four genera of the Paramyxoviridae family and indicates that the preference for replication of 6*N* length RNA genomes varies between the individual paramyxoviruses.

INTRODUCTION

Simian virus 5 (SV5) is a prototype of the *Rubulavirus* genus of the Paramyxoviridae, a diverse family of nonsegmented negative-sense RNA viruses that also includes Sendai (SeV), measles (MeV), and respiratory syncytial virus (RSV). The ~15-kb paramyxovirus genomic RNA is tightly bound by the viral nucleocapsid protein (NP) to form a nucleocapsid (NC) structure, and it is this ribonucleoprotein complex that serves as template for both replication and transcription. Two other NC-associated polypeptides, the phospho- (P) and the large (L) proteins together form the viral RNA-dependent RNA polymerase. Although the exact role of L and P in the synthesis of viral RNA is unknown, the relative abundance of the L and P polypeptides in virions (~40 and ~300 molecules per Sendai virion, respectively; Lamb *et al.,* 1976) and their distribution on the NC template (Portner *et al.,* 1988) are consistent with the proposal that L acts as the catalytic subunit of the viral polymerase, while P functions both in conjunction with L and in a noncatalytic role in RNA synthesis (Horikami *et al.,* 1992). The L and P polypeptides are required for both transcription of the genomic RNA to produce viral mRNAs and replication to produce progeny virus genomes (Hamaguchi *et al.,* 1983; Moyer and Horikami, 1991). A critical feature distinguishing transcription from genome replication is that during replication, RNA synthesis is tightly coupled to assembly of the genome with NP (Lamb and Kolakofsky, 1996).

The viral polymerase is thought to initiate RNA synthesis by binding to the 3′ end of the genomic (− sense) and antigenomic (+ sense) RNAs. As such, sequences located at the ends of the paramyxovirus RNA genome serve as promoters for replication and transcription. The importance of genomic 3′ and 5′ ends in directing paramyxovirus RNA synthesis is evident in the structure of defective interfering (DI) RNA genomes. These subgenomic RNAs contain various deletions or rearrangements of the internal segments of the viral genome and the resulting genome structures are characteristic of an individual DI RNA. However, all naturally occurring DI genomes that are replication-competent retain either the

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genomic 3′ and 5′ ends in the case of internal deletion DI RNAs or two complementary copies of the genomic 5′ end for copyback DI RNAs (reviewed in Perrault, 1981). For the SV5 genome, nucleotide sequence analysis has shown that these genomic end segments consist of a 55-base 3′ leader (le) sequence and a 31 base 5′ trailer (tr) sequence (Parks *et al.,* 1992).

The nucleotide sequence of the le and tr regions of the paramyxoviral RNA appears to be a primary determinant of the efficiency of replication to produce antigenomes and genomes, respectively (Calain and Roux, 1995; Tapparel and Roux, 1996). However, in addition to sequence determinants, recent mutational analyses of SeV DI RNAs has shown that the overall length of the viral genome can profoundly affect the efficiency of replication. This genome length requirement, called the "rule of six," dictates that efficient replication of a SeV genome will only occur when the total number of nucleotides in the genome is a multiple of six (Calain and Roux, 1993). This surprising requisite is thought to reflect the precise nature with which the genomic RNA must be encapsidated by NP to form a NC template for the viral polymerase (Calain and Roux, 1993; Pelet *et al.,* 1996). An analysis of SeV nucleocapsids by electron microscopy has indicated that each SeV NP molecule contacts six bases of genomic RNA (Egelman *et al.,* 1989). During replication, assembly of the viral genome with NP is thought to initiate with the 5′ end of the nascent chain and proceed in a 5′ to 3′ direction (Lamb and Kolakofsky, 1996). As such, a genome whose length is a multiple of six nucleotides will be precisely encapsidated by NP. The preference for replication of SeV genomes of 6*N* length has been interpreted to indicate that the viral polymerase initiates replication most efficiently when the last six nucleotides of the 3′ end promoter are bound completely by a single NP, with no unencapsidated nucleotides protruding from the 3′ end of the nucleocapsid (Pelet *et al.,* 1996).

Until recently, the *cis*- and *trans*-acting factors that direct paramyxovirus RNA synthesis have been largely ill-defined due to the lack of an experimental system by which the genomic RNA or viral proteins could be analyzed. The development of "reverse genetics" approaches to the study of negative-strand RNA virus replication have provided powerful methods to analyze important features of the viral genome that control RNA synthesis. These experimental systems have reconstituted viral replication and transcription from cDNAs corresponding to negative strand virus genome analogs (e.g., Collins *et al.,* 1991; Park *et al.,* 1991; Conzelmann and Schnell, 1994; Sidhu *et al.,* 1995), DI genomes (e.g., Pattnaik *et al.,* 1992; Calain and Roux, 1993), and full-length standard viral genomes (Schnell *et al.,* 1994; Collins *et al.,* 1995; Garcin *et al.,* 1995; Lawson *et al.,* 1995; Radecke *et al.,* 1995). While many general aspects of viral transcription and replication are shared between the negative strand RNA viruses, significant differences in the requirement for *cis*- and *trans*-acting factors between the individual viruses have become evident through use of these reverse genetics approaches.

Recent results have shown that the replication advantage seen in the case of SeV for 6*N* length genomes is not a property of all paramyxoviruses. In addition to SeV, the rule of six appears to govern the replication of MeV DI genomes (Sidhu *et al.,* 1994), but the replication of RSV genome analogs is not similarly constrained (Samal and Collins, 1996). In this report, we describe experiments to determine the affect of genome length on SV5 RNA replication. Two naturally occurring DI genomes were used as model templates in a reverse genetics system in which these DI RNAs were replicated *in vivo* entirely by cDNA-derived components. A systematic analysis indicated that while SV5 DI RNA replication was most efficient when the overall genome length was a multiple of six, the stringency of this replication requirement for SV5 genomes appeared to be less than that found previously for other paramyxoviruses.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of A549, Vero, and MDBK cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Infections of Vero and MDBK cells with the W3 strain of SV5 were as described previously (Paterson *et al.,* 1984). Vaccinia virus vTF7.3 (Fuerst *et al.,* 1987) was grown and titered in CV1 cells.

Generation and cDNA cloning of DI RNA genomes

Dishes of Vero and MDBK cells (6 cm) were infected with undiluted W3 stock of SV5 in DMEM containing 0.75% BSA. After incubation at 37° for 48 hr, extracellular media was clarified (900 *g,* 2 min) and 40% of the resulting sample was used to infect fresh duplicate monolayers of the two respective cell lines through 15 passages. To analyze subgenomic RNAs generated from these infections, fresh monolayers of the respective cell types were infected with a 1:10 dilution of each of the 15 sequential passages in DMEM containing 0.75% BSA. Total intracellular RNA was harvested 48 hr postinfection using TRIzol Reagent (GIBCO BRL; Gaithersburg, MD) and analyzed by Northern blotting (Ausubel *et al.,* 1995) with a riboprobe specific for the 5′ end of the SV5 genome as detailed below.

cDNA clones of the DI RNA genomes were generated by a modified "two halves" approach using primers that specifically amplify copyback DI RNAs from a mixture of DI and standard virus genomes (Calain *et al.,* 1992). The 3′ ends of copyback DI genomes were converted to cDNA in a reverse transcriptase reaction using total intra-

cellular RNA from cells infected with DI-containing virus stock, a genome-sense primer, L-Pro5 (5′-CACTATCGTACTGATTTTACCAAGAAGACC-3′; L gene nucleotide positions 6416 to 6387; Parks *et al.,* 1992), and AMV RTase (Promega; Madison, WI). The resulting products were used directly as a template in a PCR with Taq polymerase (Perkin–Elmer–Cetus, Norwalk, CT), L-Pro5 primer and a genome-sense primer that is specific for the SV5 trailer sequence (SM-tr, 5′-GCGTCGACACCAAGGGGAAAACCAAGATTAATCC-3′; positions 6859 to 6834; Parks *et al.,* 1992; *Sal*I site underlined). After 25 cycles (95° for 30 sec, 58° for 1 min, 72° for 3 min), the resulting PCR products were treated with T4 DNA polymerase, kinased, and ligated into the *Sma*I site of pGem3 to create the pDI3′ series.

Nucleotide sequence analysis of the 3′ end cDNAs revealed the L gene nucleotide positions comprising crossover junctions for a series of DIs and allowed the design of oligonucleotides that would specifically amplify the 5′ ends of these individual DI genomes. The 5′ ends of the DI genomes were converted to cDNA in a reverse transcriptase reaction using total intracellular RNA from cells infected with a DI-containing virus stock, a genome-sense primer specific for the crossover junction, and AMV RTase. For the DI_{852} and DI_{774} series, the crossover junction-specific primers were 5′-CGCGTCGACCTATTTCTTCATTAATATTAGCAAGAACG-3′ (852Jxn) and 5′ CGCGTCGACATTTATATCAATAAACTCTGTTGACAA-3′ (774Jxn; *Sal*I site underlined), respectively. The resulting products were used directly as a template in a PCR along with Taq polymerase, the appropriate junction-specific primer, and a primer specific for the genomic trailer sequence (5′-GGAGGCCTCTAGA*TAATACGACTCACTATA*GGGACCAAGGGGAAAACCAAGATTAATCC-3′; *Stu*I site underlined, T7 promoter in italics). After 25 cycles (95° for 1 min, 60° for 1 min, 72° for 3 min), the resulting PCR products were cut with *Stu*I and *Sal*I and ligated into the *Pvu*II and *Sal*I sites of pGem3term (a modified pGem3 vector containing the T7 transcription terminator sequence immediately following the SP6 promoter) to create the pDI5′ series. Nucleotide sequence analysis revealed that these cDNAs differ from the reported L gene sequence (Parks *et al.,* 1992) by a single G to A change at mRNA-sense base 6786 in the case of the DI_{852} series and a U to C change at mRNA-sense base 6707 for the DI_{774} series.

Plasmids encoding the complete DI_{852} and DI_{499} genomes were constructed by linking DNA fragments from the above pDI5′ and pDI3′ clones. The hepatitis delta virus antigenomic ribozyme (Perrota and Been, 1991) was fused to the precise 3′ end of the DI_{852} genome through the use of megaprimer PCR as described previously (Parks, 1994). The resulting fragment was digested with *Sph*I and *Eco*RV (contained within the DI genome sequence) and ligated into the corresponding sites of the pDI-3′ clones to generate pDI-3′ribo. To reconstruct the full-length DI genomes, pDI-3′ribo was digested with *Cla*I (contained within the DI genomic sequence) and *Sph*I, and the resulting DNA fragment was ligated into the corresponding sites of the pDI-5′ plasmids to yield $pDI_{852}(-)$ and $pDI_{499}(-)$. Both plasmids are transcribed by T7 RNA polymerase to produce a genome-sense (−) viral RNA. DI RNA replication by cDNA-derived components (see below) was found to be more efficient when antigenomic RNA was the primary T7-derived transcript (not shown). To construct plasmids that could be transcribed to produce antigenomic (+)-sense RNA, $pDI_{852}(-)$ and $pDI_{499}(-)$ were digested with *Sac*II, and the fragments were religated into their respective pDI backbones. Plasmids were screened for reinsertion of the DNA fragment in the opposite direction, generating $pDI_{852}(+)$ and $pDI_{499}(+)$, hereafter referred to as pDI_{852} and pDI_{499}. Transcription from the T7 promoter of each clone produces antigenomic RNA with an authentic 3′ end generated by the autocatalytic cleavage of the HDV ribozyme (data not shown) and is predicted to contain three additional 5′ nonviral guanosine residues.

Construction of plasmids encoding altered DI genomes

DI_{852} and DI_{499} derivatives with altered genome lengths were constructed by oligonucleotide-directed mutagenesis (Parks, 1994). Briefly, pDI_{852} or pDI_{499} DNA was used as a template in a PCR with *Pwo* polymerase (Boehringer Mannheim, Indianapolis, IN), a genome-sense primer L1 (5′-GCGGATCCGAATTCCAAGTCCTGCTTCACGATC-3′; *Eco*RI site underlined, L gene bases 6488 to 6514) and an mRNA-sense primer that contained 1 to 7 additional nucleotides inserted between L gene bases 6251 and 6252 (Parks *et al.,* 1992; 5′-GAGGCCTTCATCGATATC-$N_{(n)}$GAGCCAGTCGCAAC-3′; where *N* designates the insertion of one through seven nucleotides; *Eco*RV site underlined, see Fig. 4A for exact sequences inserted). The resulting PCR products were digested with *Eco*RV and either *Eco*RI or *Hin*dIII (contained in the pGem3 vector) and inserted into the corresponding sites of pDI_{852} or pDI_{499} to create pDI_{852} + 1 through +7 and pDI_{499} + 1, +3 and +5, respectively. To construct DI_{852} linker insertion mutant L4, pDI_{852} was digested with *Eco*RI, treated with the Klenow fragment of DNA polymerase in the presence of dNTPs, and religated to yield a plasmid encoding a DI genome with an insertion of four bases. pDI_{852}-L12, pDI_{852}-L20, and pDI_{852}-L36 were constructed by inserting one, two, and four copies, respectively, of an *Avr*I linker (5′-GCCTAGGC-3′) into the blunt-ended *Eco*RI site of L4. The structure of DNAs encoding the altered genomes was confirmed by nucleotide sequence analysis.

DNA plasmids

DNA plasmids containing the SV5 NP, P, and L genes under control of the T7 promoter and the expression of

plasmid-derived proteins have been described in detail previously (Paterson *et al.,* 1984; Thomas *et al.,* 1988; Parks *et al.,* 1992; Parks, 1994). To construct pUC19-NP, a *Pvu*II–*Nhe*I fragment encoding the SV5 NP protein under control of the bacteriophage T7 promoter was excised from pGEM3-NP (Parks *et al.,* 1992) and was inserted into the *Sma*I–*Xba*I sites of pUC19. The nucleotide sequence of the NP gene in the resulting plasmid (pUC19-NP3A) was determined to confirm identity with the published sequence (Parks *et al.,* 1992).

In vivo DI replication

Although subgenomic RNAs were cloned from SV5-infected Vero cells, DI genome replication directed by cDNA-derived components was found to be more efficient in A549 cells. Subconfluent 3.5-cm dishes of A549 cells were infected (m.o.i. ~5) for 1 hr with vTF7.3, a recombinant vaccinia virus that expresses bacteriophage T7 RNA polymerase (Fuerst *et al.,* 1986). Typically, infected cells were transfected with plasmids encoding the SV5 DI antigenomic constructs (0.8 μg), along with pGEM3-L (1.5–2.0 μg), pGEM2-P (0.2 μg), and pUC19-NP (2.0 μg), using Lipofectin reagent (Gibco BRL, Gaithersburg, MD) as described previously (Parks, 1994). Media contained 100 μg/ml cytosine β-D-arabinofuranoside (AraC; Sigma Chemical Co., St. Louis, MO). pGEM control plasmid was used to normalize for the total amount of transfected DNA. After 24 hr, the transfection media was replaced with serum-free DMEM containing 100 μg/ml Ara-C.

Northern blot analysis

For analysis of cDNA-derived viral RNA genomes, total intracellular RNA was harvested at 40 hr posttransfection using TRIzol reagent (Gibco-BRL, Gaithersburg, MD). Samples were treated with RNAse-free DNAse (Promega, Madison, WI) to remove residual plasmid DNA and were analyzed by electrophoresis (110 V for 2 hr) on 1.2% agarose-formaldehyde gels (Ausubel *et al.,* 1995). Ethidium bromide staining of ribosomal RNA in the gels showed little variability between the amount of RNA loaded for each sample. After capillary transfer in 10× SSC (1.5 *M* NaCl, 0.15 *M* NaCitrate, pH 7.0) to a Zeta Probe nylon membrane (0.2 μm pore; Bio-Rad, Hercules, CA), RNA was fixed by UV crosslinking.

For analyzing DI genomes produced during virus passage, a 167-nucleotide ^{32}P-labeled (+)-sense riboprobe was generated that contained nucleotides 6750 to 6859 of the SV5 L gene and trailer sequences (Parks *et al.,* 1992). For detection of internal deletion DI genomes, a 129-nucleotide ^{32}P-labeled (+)-sense riboprobe was used which contained nucleotides 90 to 172 of the NP gene sequence (Parks *et al.,* 1992). For analyzing DI_{852} and DI_{499} replication from cDNA-derived components, plasmid pGEM5-SspI was constructed such that the *Ssp*I fragment of DI_{852} was inserted into the *Eco*RV site of pGEM5. A 723-base (+)-sense riboprobe of the same polarity as the T7-derived DI_{852} genome was generated by *in vitro* transcription of *Nco*I-linearized pGEM5–*Ssp*I with SP6 RNA polymerase in the presence of [^{32}P]CTP. Membranes were hybridized with purified riboprobes in ExpressHyb (Clonetech; Palo Alto, CA) for 1 hr at 68°. After washing (15 min each in 2× SSC/0.1% SDS, 0.2× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS), the membrane was exposed at −70° to radiographic film. Quantitation of RNA replication products was performed using the AMBIS 4000 Image Acquisition system and software (AMBIS; San Diego, CA).

RESULTS

Generation of SV5 DI RNA genomes

SV5 DI RNA genomes were generated by undiluted passage of virus in two tissue culture cell lines. Monolayers of Vero or MDBK cells were infected with standard SV5, and media collected from this initial infection was used undiluted to infect fresh monolayers of cells through 15 sequential passages. To determine if subgenomic DI RNAs had been generated during these infections, aliquots from each passage were used to infect fresh monolayers of each cell type and after 2 days intracellular RNAs were harvested. RNAs were examined by Northern blot analysis using a (+)-sense riboprobe complementary to the trailer region of the standard SV5 genome, a sequence that is predicted to be present on both internal deletion and copyback types of DI genomes (Perrault, 1981). The results for both the Vero cell and MDBK cell infections are shown in Fig. 1.

A predominant subgenomic RNA was first detected in Vero cells infected with passage 4 virus (Fig. 1A, lane 4), and this RNA species was absent from samples isolated from mock-infected cells or cells infected with the standard SV5 virus stock that had been used to initiate the undiluted passage (Fig. 1A, mock and SV5 lanes, respectively). Additional distinct subgenomic RNAs ranging from ~0.8 to ~2 kb were detected in samples from cells infected with later passage virus (e.g., lanes 7, 10, and 14). The relative abundance of individual subgenomic RNAs appeared to increase and then decrease with further passage (e.g., lanes 4–6 and 7–9), a pattern that is characteristic of the reported cyclic fluctuation of DI genome replication with that of the cognate helper virus (Holland, 1987, and references therein). For a given virus stock, a very similar pattern of subgenomic RNAs was obtained by Northern blotting of RNA recovered from RNP complexes that had been banded on CsCl gradients, consistent with these forms being *bona fide* replication-competent SV5 subgenomic RNAs (data not shown).

By contrast to the above results obtained in Vero cells, undiluted passage of SV5 in MDBK cells resulted in only one predominant subgenomic RNA that could be de-

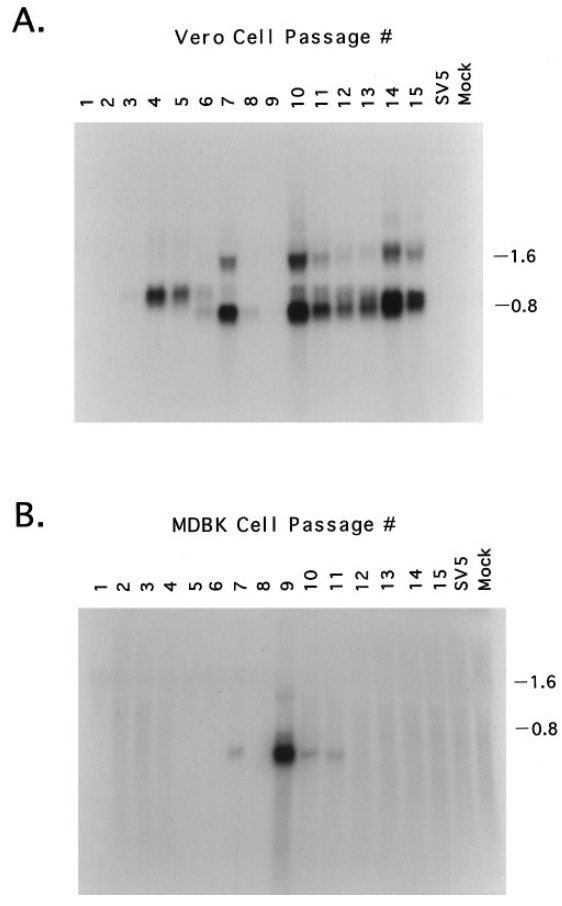


FIG. 1. SV5 subgenomic RNAs are generated by sequential undiluted passage in two cell types. SV5 was passaged through 15 sequential undiluted infections of Vero (A) or MDBK (B) cells as described under Materials and Methods. Fresh monolayers of the respective cell types were infected with an aliquot of media collected from each of the 15 sequential passages, and total intracellular RNA was harvested 48 hr postinfection. Samples were analyzed by Northern blotting with a (+) sense riboprobe complementary to the trailer sequences located at the 5′ end of the SV5 genome. Lanes marked mock and SV5 represent samples from mock-infected cells and cells infected with the standard SV5 virus stock that had been used to initiate the undiluted passage. Numbers indicate approximate size of marker RNAs in kilobases.

tected by Northern blotting (Fig. 1B, lane 9). This ~750-base RNA was first seen in MDBK cells infected with pass 7 virus stock, was relatively abundant by pass 9, and was undetectable by pass 12 (lanes 7, 9, and 12). Both Vero and MDBK cells are permissive for SV5 replication and growth, with the yield of standard virus being higher from infections of MDBK cells (not shown). The reason for the differences between these two cell types in the ability to support the *de novo* generation or amplification of detectable levels of subgenomic RNAs is unclear. However, it is known that cell type can profoundly influence DI RNA generation and replication (Perrault, 1981; Holland, 1987).

The above results obtained with a trailer-specific riboprobe indicated that subgenomic RNAs had been generated during undiluted passage, but they could not distinguish between copyback type (two complementary copies of 5′ trailer) and internal deletion type (one copy each of 3′ leader and 5′ trailer) DI RNAs. Northern blotting of RNAs from cells infected with the DI-containing virus stocks using a riboprobe specific for the 3′ NP region of the SV5 genome did not reveal subgenomic RNAs (data not shown), suggesting that either internal deletion DI genomes were not generated in these infections or the level of replication of this type of DI genome was below the limit of detection by Northern blotting. Virus derived from Vero pass 14-infected cells was chosen for subsequent analysis, as this stock appeared to generate the most diverse sizes of subgenomic RNAs, and the MDBK-derived stock containing the major ~750-base RNA was analyzed in parallel.

cDNA cloning of SV5 DI genomes

To further characterize the SV5 subgenomic RNAs, cDNA clones encoding DI genomes were generated by a modified "two-halves" approach (Calain *et al.,* 1992). Total intracellular RNA derived from infection of the respective cell types with Vero cell pass 14 virus and the MDBK-derived virus stocks was isolated. cDNAs encoding the 3′ end of the subgenomic RNAs were generated in an RT-PCR using two genome-sense primers that specifically amplify copyback types of DI RNAs (see Materials and Methods, and Calain *et al.,* 1992). Clones encoding the 3′ ends of nine Vero pass 14-derived subgenomic RNAs and of the predominant MDBK-derived DI genome were isolated and their nucleotide sequence was determined. Primers specific for two of these subgenomic RNAs (DI_{852} and DI_{834}) were used to generate cDNAs encoding DI RNA 5′ ends. Nucleotide sequence analysis of the cDNAs revealed the overall sizes of the individual genomes, the specific crossover junction linking genomic and antigenomic sequences, and the extent of complementarity (or stem length) between the terminal 5′ and 3′ ends of each DI genome.

As shown in Table 1, the overall sizes of the cloned Vero pass 14-derived DI genomes ranged from 449 to 1365 bases in length (DI_{449} and DI_{1365}). A comparison of these genome lengths with the mixed population of subgenomic RNAs detected by Northern blotting (Fig. 1A) indicated that many of these clones encoded genomes that were not abundantly represented in cells infected with the Vero pass 14 virus stock (e.g., DI_{449} and DI_{499}), while other species (e.g., ~800 base species) appeared to be major subgenomic RNAs in the population. The overall lengths of all natural SeV genomes, both standard and DI, are multiples of six (the 6*N* requirement, Hausmann *et al.,* 1996). The single DI genome cloned from MDBK-derived stock virus (DI_{744}) was found to have a 6*N* length (Table 1 and schematic in Fig. 2A). However, only three of the nine DI RNA genomes cloned from Vero cells were found to have an overall 6*N* genome length (DI_{774},

TABLE 1

Characteristics of SV5 DI RNA Genomes

DI	Cell type[a]	Crossover junction[b]	Terminal complementarity[c]	Genome size[d]	6N?[e]
DI_{744}	M	6226–6750	110	744[f]	Yes
DI_{449}	V	6121–6747	113	449	No
DI_{499}	V	6121–6747	113	499	No
DI_{698}	V	6242–6780	80	698[f]	No
DI_{774}	V	6166–6725	135	774	Yes
DI_{834}	V	6166–6725	135	834	Yes
DI_{852}	V	6121–6747	113	852	Yes
DI_{1149}	V	6004–6567	293	1149[f]	No
DI_{1363}	V	5986–6371	495	1363[f]	No
DI_{1365}	V	5990–6365	489	1365[f]	No

[a] Cell type from which the DI particle genome was isolated; V, Vero; M, MDBK.

[b] SV5 L gene nucleotide positions within the DI antigenome that compose the crossover junction linking antigenomic (first number) to genomic (second number) sequences. Both numbers refer to mRNA sequence (Parks *et al.,* 1992).

[c] Number of bases of uninterrupted complementarity between 3′ and 5′ ends of the genome.

[d] Overall size of the DI genome derived by nucleotide sequence analysis of overlapping 5′ and 3′ end cDNAs.

[e] Status of the genome length with regard to the "rule of six" (see text).

[f] Predicted overall size derived by nucleotide sequence analysis of 3′ end cDNAs and assuming 5′ end sequence identity with the standard viral genome.

DI_{834}, and DI_{852}). The remaining DI genomes with sizes that are not a multiple of six were longer than 6*N* length by 1, 2, 3, or 5 residues.

The crossover junction of a copyback DI RNA is thought to represent the positions in the standard virus genome where the polymerase had disengaged from the template during replication and reattached to the nascent RNA strand to continue replication (Perrault, 1981). As shown in Table 1, DI genomes cloned from Vero pass 14-infected cells had crossover junctions that were not well conserved, suggesting that there were no copyback "hot spots" for departure and reentry on the SV5 genome as proposed previously for MeV DI genomes (Sidhu *et al.,* 1994). A striking feature of this sequence analysis is the range of complementarity between the terminal 5′ and 3′ ends of the individual SV5 copyback DI genomes, and these values ranged from 80 to 495 bases. The latter value is much larger than that reported previously for naturally occurring copyback genomes of other negative-stranded RNA viruses. For VSV, this range is reported to be 45 to 150 bases (Holland, 1987); for SeV, 100 to 210 bases (Mottet and Roux, 1989; Re, 1991) and for MeV, 95 to 229 bases (Sidhu *et al.,* 1994).

Nucleotide sequence analysis indicated that many of the Vero pass 14-derived copyback DI genomes contained identical crossover junctions and extent of terminal complementarity (e.g., DI_{449}, DI_{499}, DI_{852}, and DI_{834}, DI_{774}), but these genomes differed in overall length. As shown schematically in Fig. 2, these RNAs could be grouped into two distinct nested sets of DI genomes. Members of the DI_{852} set (including DI_{852}, DI_{499}, and DI_{449}) each contained the same crossover junction (anti-genomic bases 6121 and 6747) and had 113 bases of terminal complementarity, but DI_{499} contained a 353-base deletion from within the 3′ end of the L gene sequence (nucleotide positions 6393 to 6745), and DI_{449} was further truncated by a 50-base deletion (positions 6252 to 6301; Fig. 2B). Members of the DI_{834} set (including DI_{834} and DI_{774}) contained a common crossover junction that was distinct from those in the DI_{852} series and shared 135 bases of terminal complementarity, but differed from each other by an internal deletion of 60 bases in the case of DI_{774}. Surprisingly, the two cloned genomes comprising the DI_{834} set contained a stretch of nontemplated adenosine (A) residues inserted precisely at the crossover junction (antigenome-sense; see Fig. 2B). It is noteworthy that the net effect of the insertion of five nontemplated A residues into DI_{834} was to create a genome that was 6*N* in length. It is unlikely that the insertion of these nucleotides is an anomaly of the RT-PCR cloning procedure, since the DI_{834} genome was cloned a second time from an independent preparation of nucleocapsid-associated RNA from cells infected with Vero pass 14 virus stock (not shown). The finding of two nested sets of DI RNA genomes suggests that DI_{852} and DI_{834} represent two principle DI RNAs that formed during replication of the standard virus genome and that subsequent deletions occurred during the amplification of these "parental" DI RNAs.

Replication of an SV5 copyback DI genome *in vivo* from cDNA-derived components

As a first step in establishing a system for the analysis of SV5 DI RNA replication, cDNA clones encoding full-length DI_{852} and DI_{499} genomes were inserted into plasmids between the promoter for T7 RNA polymerase and the antigenomic sequences from the self-cleaving HDV ribozyme (Fig. 2C, T7p and HDV, respectively), as described previously (Pattnaik *et al.,* 1992). It was predicted that transcription from the T7p would generate (+)-sense RNAs containing three additional 5′ guanosine (G) residues and an exact 3′ end due to ribozyme self-cleavage (Pattnaik *et al.,* 1992; Perrota and Bean, 1991).

To determine if cDNA-derived L, P, and NP proteins could function in replicating the DI_{852} RNA genome, monolayers of A549 cells were first infected with vTF7.3, a recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase (Fuerst *et al.,* 1987). These infected cells were then cotransfected with plasmids encoding the DI_{852} antigenome, L, P, and NP proteins, all of which were under control of the T7p. Northern blot analysis of total intracellular RNA with a strand-specific riboprobe was used to determine if the plasmid-derived viral proteins could support

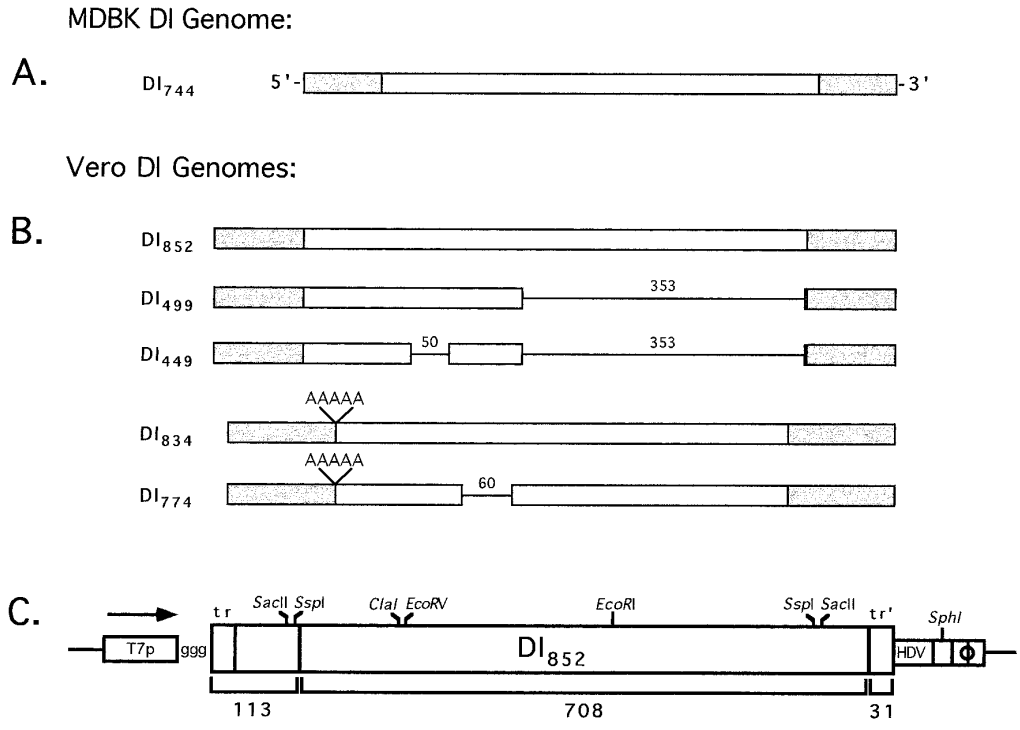


FIG. 2. Structure of cloned SV5 copyback DI genomes. SV5 DI RNAs cloned from infected MDBK (A) and Vero cells (B) are shown schematically as rectangles with the 5′ (left) and 3′ (right) ends of the antigenomic RNA indicated. Shaded boxes and lines denote the extent of terminal complementarity and genome deletions, respectively. Numbers correspond to the size of the deleted segments. For DI_{834} and DI_{774}, five nontemplated A residues present at the crossover junction are indicated. (C) Plasmid DNA encoding the DI_{852} antigenome is shown schematically as a rectangular box flanked on the left (5′ end) by the bacteriophage T7 RNA polymerase promoter (T7p) and on the right (3′ end) by the self-cleaving HDV ribozyme and T7 transcription terminator (ø). The number of nucleotides composing the 5′ region of terminal complementarity (113 bases), the 3′ end of the L gene (708 bases) and the 31 base anti-trailer sequences are shown below the box. Relevant restriction sites used in subcloning, mutagenesis, and to generate riboprobes for Northern blotting are indicated. The horizontal arrow indicates the direction of transcription from the T7p to produce a (+) sense DI RNA antigenome containing three non-viral G residues (ggg) at the 5′ end. tr, genomic trailer sequences; tr′, antigenomic trailer sequences.

conversion of the plus-sense DI_{852} antigenome generated by T7 polymerase to the minus-sense complementary strand. The probe chosen for use in this assay was of the same polarity (plus-sense) as the T7-derived DI_{852} RNA antigenome, so that only complementary minus-sense genomes would be detected.

Titration experiments were carried out to determine the optimum level of SV5 plasmids that would support replication of the DI_{852} antigenome. Cells were transfected with a constant level of the L-, P-, and DI_{852}-encoding plasmids, and increasing amounts of the NP plasmid. As shown in Fig. 3A, RNA from cells cotransfected with increasing amounts of the NP plasmid showed a dose-dependent increase in a major ~0.9-kb RNA species that was complementary to the T7 polymerase-derived DI_{852} antigenome (lanes 1–7). The electrophoretic mobility of this RNA matched that of a major DI RNA seen in the marker lane, which represents a sample of RNA from cells infected with the virus stock from which DI_{852} had been cloned (lane M). An optimum level of transfected NP plasmid was seen between ~1.5–2.0 μg and above this level DI_{852} replication was decreased (lane 8). Synthesis of RNA complementary to the input DI_{852} antigenome was not detected in samples of transfected cells in which DNA encoding a mutant form of the SV5 L protein that lacks two highly conserved L protein domains (Parks, 1994) was substituted in place of the WT L plasmid (ΔL, lane 9). Likewise, RNA samples from transfected cells in which the P or DI_{852} plasmids were excluded did not contain the ~0.9-kb RNA species (lanes 10 and 11, respectively). In cases where NP plasmid was omitted or was cotransfected at low levels, a smaller, more diffusely migrating RNA was present (lanes 1–3). These smaller RNAs are not detected when DI_{852} replication products are banded on CsCl gradients before Northern blot analysis (not shown). In addition to the ~0.9-kb DI_{852} replication product, a ~1.8-kb RNA species of unknown origin was detected (lanes 5–7). Together, these data indicate that the SV5 plasmid-derived L, P, and NP proteins are functional and are the minimal viral components needed to support the *in vivo* replication of the DI_{852} antigenome.

Titrations of the amount of transfected DNA encoding the P protein showed an increase in DI_{852} replication with increasing levels of transfected P plasmid between 0.1 and 0.4 μg, while addition of 0.8 μg or more of P plasmid

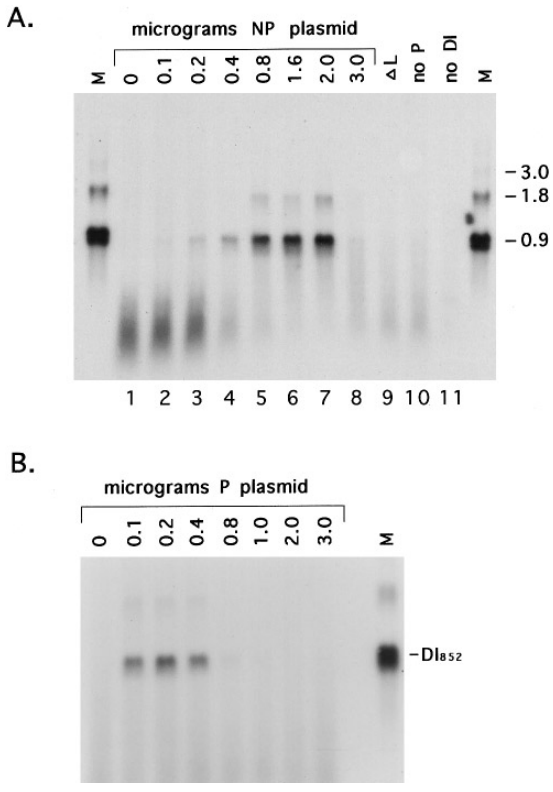


FIG. 3. Replication of the DI_{852} genome by cDNA-derived components. (A) Titration of NP plasmid levels. A549 cells infected with vaccinia virus vTF7.3 were cotransfected with plasmids encoding the SV5 P protein (0.2 μg), L protein (1.5 μg), and DI_{852} genome (0.8 μg) along with the indicated amounts of a plasmid encoding NP. Total intracellular RNA was analyzed by Northern blotting with a ^{32}P-labeled riboprobe of the same polarity as the T7-derived DI_{852} antigenome. M denotes a marker lane of RNA from cells infected with the SV5 virus stock from which the DI_{852} genome had been cloned. Control samples in which either a mutant form of L had substituted for the wt L (ΔL; Parks, 1994) or plasmids encoding DI_{852} and the P protein had been omitted are shown on the right. (B) Titration of P plasmid levels. vTF7.3-infected A549 cells were transfected with plasmids encoding the SV5 L protein (2.0 μg), NP protein (2.0 μg), and DI_{852} genome (0.8 μg) along with the indicated amounts of P plasmid. Samples were analyzed by Northern blotting as described for A.

had an inhibitory effect on replication (Fig. 3B). Titrations of L plasmid showed a broad optimum in the replication assay with maximum signal detected when 1.5 to 2.0 μg of L plasmid was transfected (not shown).

Replication of SV5 DI genomes is enhanced when the overall genome length is a multiple of six

To determine if the overall length of an SV5 DI genome influenced replication efficiency, a series of plasmids were constructed that encoded DI_{852} derivatives having one to seven additional nucleotides inserted near the *Eco*RV site (Fig. 4A, and see Fig. 2C for location within DI_{852}). Cells infected with vTF7.3 were transfected with L, P, and NP plasmids along with DNA encoding either the 6*N* length DI_{852} (WT) RNA or one of the insertion mutants. Intracellular RNA was analyzed by Northern blot using a (+)-sense riboprobe as described above. An autoradiogram from a representative experiment is shown in Fig. 4B. DI_{852} derivatives containing one to four nucleotide insertions (Fig. 4B, lanes 1–4) were found to replicate to levels that were ~35–50% of that seen for the unaltered 6*N*-length DI_{852} (lane 0). An insertion of five residues partially restored replication to 75% that of the WT genome. However, replication levels did not match that of the WT DI_{852} genome until a total of six nucleotides had been inserted. Thus, the progressive addition of one to six nucleotides led to an initial reduction in DI_{852} replication (+1 to +4 bases), followed by a progressive increase back to WT levels (+5 and +6). Replication was again reduced to levels similar to that of the +1 to +4 mutants when the genome length was further increased by inserting a total of seven residues (compare lanes 1–4 to lane 7, Fig. 4B).

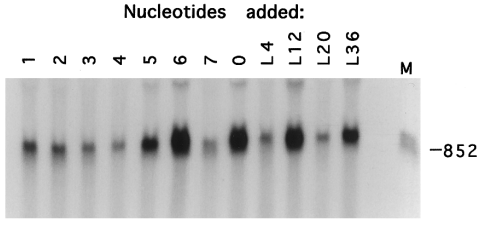
A second set of plasmids was constructed to determine if length alterations introduced at a different point in the genome had a similar effect on DI_{852} replication. DNA linkers were inserted into the *Eco*RI site of the DI_{852} plasmid (see Fig. 2C for location within DI_{852}), and this resulted in mutants that contained an additional 4, 12, 20, and 36 nucleotides (L4, L12, L20, and L36, Fig. 4A). When assayed for replication by cDNA-derived components, a DI_{852} derivative containing a 12-base insertion was replicated to a level closely matching that of the unaltered WT genome (lane L12, Fig. 4 and quantitated in 4C), while insertions of 4 and 20 residues reduced replication to 41 and 25%, respectively. Although mutant L36 replicated to higher levels than the L4 and L20 genomes, amplification of this derivative was consistently lower than that of the WT DI_{852} genome (60–70%). It is possible that extensive secondary structure introduced into the genome by the insertion of four tandem linkers has interfered with either encapsidation of the RNA genome or with the activity of the NC template. From three independent experiments, the mean relative replication level of each of the DI_{852} insertion mutants was calculated and the results are shown graphically in Fig. 4C. These results indicate that the efficiency of DI_{852} replication is highest when the genome length is a multiple of six.

The DI_{852} and DI_{499} genomes contain the same crossover junction and extent of terminal complementarity, but differ by an internal deletion in DI_{499} (see Table 1 and schematic in Fig. 2B). When assayed using cDNA-derived components, DI_{499} replication was low relative to the parental DI_{852} genome (data not shown), a result that is consistent with the inability to detect a 499-base DI genome by Northern blotting of RNA from cells infected with the virus stock from which DI_{499} was cloned (see Fig. 1A). It is possible that sequences which were deleted during the generation of DI_{499} contained essential *cis*-acting elements necessary for efficient replication. Alternatively, the low replication efficiency of the naturally occurring DI_{499} RNA could be a consequence of genome

A.

# Bases inserted	Sequence	6N?
0	WT	Yes
1	A	No
2	AT	No
3	ATA	No
4	ATAT	No
5	ATATA	No
6	ATATAT	Yes
7	ATATATA	No
4	AATT	No
12	AATT(GCCTAGGC)	Yes
20	AATT(GCCTAGGC)$_2$	No
36	AATT(GCCTAGGC)$_4$	Yes

B.



C.

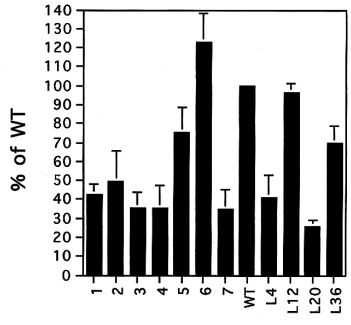


FIG. 4. Replication of the DI_{852} genome is enhanced when the overall genome length is a multiple of six. (A) The number and nucleotide sequence of the bases inserted to create DI_{852} derivatives with altered genome lengths is listed. WT indicates the wild-type DI genome sequence. (B) Results from a representative assay for the replication of DI_{852} derivatives. vTF7.3-infected A549 cells were cotransfected with plasmids encoding NP (2 μg), P (0.2 μg), L (2 μg), and a DI_{852} genome derivative (0.8 μg) containing the indicated number of inserted nucleotides. Total intracellular RNA was analyzed by Northern blotting with a ^{32}P-labeled riboprobe of the same polarity as the T7-derived DI_{852} antigenome. M denotes a marker lane of RNA from cells infected with the SV5 DI virus stock from which the DI_{852} genome had been cloned. (C) Quantitation of RNA replication for the DI_{852} mutants. The level of replication for altered DI_{852} genomes was determined from three independent experiments and is expressed as a percentage of that determined for the WT DI_{852} genome done in parallel. Bars represent the standard deviation from the mean.

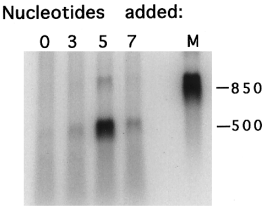
length, since the overall size of this RNA is not a multiple of six.

To determine if the efficiency of DI_{499} replication could be enhanced by genome length alterations, three DI_{499} derivatives were constructed such that the viral genomes contained insertions of three, five, or seven nucleotides (Fig. 5A). vTF7.3-infected cells were transfected with plasmids encoding L, P, and NP along with plasmids encoding DI_{499} or a length-altered derivative. Intracellular RNA was analyzed by Northern blotting using a (+)-sense riboprobe as described above. An autoradiogram from a representative experiment is shown in Fig. 5B. The WT DI_{499} genome replicated to very low levels that were only detected with very long exposure times (Fig. 5, lane O). An insertion of five nucleotides into the DI_{499} genome created a genome length that was a multiple of six (504 total bases) and led to a ~10-fold stimulation of replication over that of the WT DI_{499} level (compare lanes 0 and 5, Fig. 5B). Interestingly, although the addition of three or seven residues to the DI_{499} genome did not create RNAs of 6*N* length, replication of the DI_{499} + 3 and DI_{499} + 7 genomes was also enhanced over the WT level (1.6- and 2.2-fold), but this stimulation was much lower than that seen for the +5 (6*N*) genome. The results from three independent replication assays (Fig. 5C) indicate that DI_{499} replication is stimulated by genome insertions that create an overall 6*N* length. DI_{499} replication

A.

# Bases inserted	Sequence	6N?
0	WT	No
3	ATA	No
5	ATATA	Yes
7	ATATATA	No

B.



C.

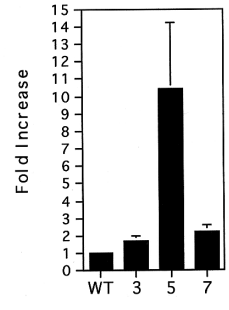


FIG. 5. Replication of the DI_{499} genome is enhanced by insertions that increase the genome length to a multiple of six. (A) The number and nucleotide sequence of the bases inserted to create DI_{499} derivatives with altered genome lengths is listed. WT indicates the wild-type DI genome sequence. (B) Results from a representative assay for the replication of DI_{499} derivatives. vTF7.3-infected A549 cells were cotransfected with SV5 plasmids and total intracellular RNA was harvested and analyzed as described in the legend to Fig. 4. (C) Quantitation of RNA replication for the DI_{499} mutants. The level of replication for altered DI_{499} genomes was determined from three independent experiments. The results are expressed as the fold increase over replication levels obtained for WT DI_{499} done in parallel. Bars represent the standard deviation from the mean.

appeared to be more sensitive to changes in the 6*N* genome length than did DI_{852} (maximum 10.4-fold stimulation versus ~2 to 3-fold reduction, respectively). Whether this difference reflects another factor, such as overall size, is unknown at this time.

The 5′ end of the primary T7 polymerase-derived SV5 DI RNA transcript is predicted to contain three nonviral G residues that contribute to efficient *in vivo* transcription by T7 RNA polymerase. Plasmids were designed such that T7 polymerase initiated RNA synthesis on the authentic 5′ genomic A residue, but *in vivo* T7 polymerase-derived RNA transcripts from these plasmids or the corresponding replication products were not detected (not shown). In the case of SeV and VSV, primer extension data have indicated that nonviral 5′ G residues on internal deletion-type DI RNA genomes are not retained during replication by the viral L polymerase (Cadd *et al.,* 1996; Pattnaik *et al.,* 1992). Attempts to determine the status of the SV5 DI_{852} 5′ end by primer extension or by approaches involving RNA circularization and RT-PCR (Samal and Collins, 1996) were unsuccessful, perhaps reflecting extensive hybridization between the complementary 3′ and 5′ ends of the copyback genome (113 bases). We have constructed an SV5 internal deletion type genome which, like the copy-back genomes, contains three 5′ end nonviral G residues resulting from the T7 polymerase. Primer extension analysis of NC-associated RNA produced during replication of this internal deletion genome revealed RNAs that contained the authentic 5′ end of the genome (not shown). Thus, extending the results from replication of the internal deletion type genome to the amplification of the copy-back DI genomes analyzed here provides indirect evidence, suggesting that 5′ end nonviral G residues are not retained during amplification of the DI_{852} and DI_{499} genomes.

DISCUSSION

As part of an effort to analyze genomic signals directing paramyxovirus RNA synthesis, DI genomes of the prototype *Rubulavirus* SV5 were produced by undiluted passage of virus in both MDBK and Vero cells. Undiluted passage of SV5 in these two cell types had very different outcomes. While a spectrum of DI genomes that could be detected by Northern blotting was produced by undiluted passage of SV5 in Vero cells, similar infections of MDBK cells produced only a single predominant subgenomic RNA that was rapidly lost during subsequent passage. These results support the contention that the generation and/or amplification of RNA virus DI genomes is greatly affected by host cell factors (Perrault, 1981; Holland, 1987), and that infections that result in efficient replication and large yields of standard virus (e.g., SV5 infections of MDBK cells) will not necessarily generate abundant DI genomes, even during undiluted passage.

Internal deletion and copyback types of DI genomes are thought to be generated via a mechanism in which the viral polymerase complex disengages from the RNA template and resumes synthesis either downstream on the original template or on the nascent RNA chain it is still carrying, respectively (Leppert *et al.,* 1977). The detection of only copyback-type DI genomes by undiluted passage of SV5 was not unexpected. For most of the Paramyxoviridae, copyback RNAs are the most common type of DI genome (Perrault, 1981; Sidhu *et al.,* 1994); however, for SeV, both copyback types and internal deletion types can be found (Re, 1991, and references therein). It is interesting to note that two distinct nested sets of DI genomes were detected in the Vero cell-derived virus stock used in these studies, and these sets of related genomes included a "parental" copyback RNA and "progeny" genomes in which various internal segments had been deleted. Numerous attempts to identify internal deletion type SV5 DI RNA genomes were without success. The presence of a series of copyback DI RNA genomes with internal deletions suggests that the SV5 polymerase may be capable of generating a DI genome of the internal deletion type, but if generated, these genomes may not compete effectively with the abundant copyback DI RNAs that predominate in the population (Calain and Roux, 1995; Holland, 1987).

For one DI RNA nested set (the DI_{852} series), the internal deletion common to the two progeny genomes extended to within two bases of the 5′ end (genome-sense) inverted repeat, suggesting that important *cis*-acting sequences are contained within the 113 5′-proximal nucleotides. The identification of a 698-base SV5 copyback DI genome having 80 bases of terminal complementarity (DI_{698}, Table 1) represents the shortest stem region yet reported for a naturally occurring paramyxovirus DI RNA (Calain and Roux, 1995; Pelet *et al.,* 1996). The DI_{698} genome is not an abundant species in cells infected with Vero pass 14 virus as assayed by Northern blot analysis, and the relative ability of this individual DI to replicate is unknown. Nevertheless, the structure of DI_{698} suggests that the minimal SV5 antigenomic promoter can be further narrowed to the 5′-proximal 80 bases. In the case of a copyback SeV DI, a reverse genetics analysis has localized the minimal antigenomic replication promoter to the terminal 31 bases of the viral RNA (Tapparel and Roux, 1996). Work is in progress to map the minimal sequences directing efficient replication of the SV5 DI RNA genomes described here.

The overall lengths of all natural SeV standard and DI RNA genomes are multiples of six (the 6*N* requirement; Hausmann *et al.,* 1996). In the case of MeV copyback DI genomes that were isolated from brain samples of SSPE patients, nucleotide sequence analysis showed that the majority of cloned DI RNAs had an overall genome length that was a multiple of six, like that of the standard MeV genome (Sidhu *et al.,* 1994; 1995), but non-6*N*-length DI RNA genomes were also identified (4 of 20 RNAs). In the case of the SV5 DI-containing virus stocks analyzed in

the work reported here, only 4 of the 10 cloned SV5 genomes were of 6*N* length (DI_{744}, DI_{774}, DI_{834}, and DI_{852}; Table 1). With the caveat that the relative abundance of the individual cloned MeV and SV5 DI RNA genomes within the mixed populations have not been determined, these data suggest that the 6*N* genome length requirement of naturally occurring DI RNAs may vary among these paramyxoviruses.

An experimental system has been developed whereby an SV5 copyback DI RNA genome can be replicated *in vivo* entirely by cDNA-derived components. As with other negative-strand RNA viruses, the L, P, and NP proteins were found to be necessary and sufficient to direct RNA synthesis from an SV5 DI RNA template (Lamb and Kolakofsky, 1996). This reverse genetics system has allowed a systematic analysis of the influence of 6*N* genome length on the replication of two naturally occurring DI RNAs that have different overall lengths but identical crossover points and terminal complementarity. The results of these experiments indicate that the replication of the SV5 DI RNAs that were of 6*N* length was enhanced over that of the non-6*N* counterparts. The relative effect on replication appeared to differ for alterations that disrupted (for the DI_{852} RNA) or created (for the DI_{499} RNA) 6*N* genome lengths. While previous work has shown that smaller DI RNAs do not necessarily replicate more efficiently than larger DI RNAs (Perrault, 1981; Holland, 1987), it is possible that the greater effect of alterations to the non-6*N* DI_{499} RNA (maximum ~10-fold stimulation) compared to the 6*N* DI_{852} RNA (maximum ~5-fold reduction) simply reflects the smaller size of the DI_{499} genome. The increased efficiency of replication that resulted by creating 6*N* length to the DI_{499} genome suggests that the sequences that were deleted during generation of the DI_{499} genome from the parental DI_{852} RNA are not essential for replication and that the lack of 6*N* length may have been a major factor determining the relative abundance of the individual DI RNAs in the mixed population from which these genomes had been cloned.

The work reported here completes the testing of the rule of six replication requirement for representatives of each of the four genera of the Paramyxoviridae family (*Paramyxo*-, *Morbilli*-, *Rubula*-, and *Pneumovirus;* Lamb and Kolakofsky, 1996), and illustrates differences between members of this diverse family of viruses. Using DI genomes of both internal deletion and copyback types, the *Paramyxovirus* genus prototype SeV was found to be highly dependent on a 6*N* genome length for efficient replication (Calain and Roux, 1993, 1995; Pelet *et al.,* 1996; Hausmann *et al.,* 1996). Likewise, when enzyme activity generated from a synthetic minigenome expressing CAT mRNA was used as an indirect assay, replication of the *Morbillivirus* MeV genome was reported to be sensitive to changes in 6*N* length (Sidhu *et al.,* 1994). By contrast, the more distantly related *Pneumovirus* RSV showed no particular replicative advantage to genome analogs having any of the integer genome lengths tested (Samal and Collins, 1996). The systematic analysis reported here for replication of the *Rubulavirus* prototype SV5 revealed a situation between the two extremes seen with RSV and SeV genomes. Unlike RSV genome analogs, there was a clear influence of 6*N* genome length on SV5 DI RNA replication, but the stringency of this replication requirement appeared to be less than that of SeV DI genomes. SeV DI replication levels can be reduced by as much as 20-fold by insertions that disrupt overall 6*N* length (Calain and Roux, 1993; Pelet *et al.,* 1996). For SV5, genome insertions of one to four bases reduced replication by only ~3 to 5-fold, and DI RNA genomes with a five base insertion replicated to ~70% of the WT level. As all of these data on SV5 replication were obtained using derivatives of a copyback DI template, it will be of interest to determine if the apparently less stringent requirement for SV5 6*N* genome length also applies to the replication of an internal deletion DI genome. Taken together, results from the cDNA cloning of naturally occurring SV5 DI genomes and the mutational analysis using the reverse genetics system suggest that 6*N* genome length influences SV5 DI RNA replication, but the stringency of this replication requirement for SV5 appears to be less than that found previously for other paramyxoviruses.

During transcription of the paramyxovirus genome, two distinct steps involve the addition of nontemplated nucleotides by the viral polymerase (Jacques and Kolakofsky, 1991). A short stretch of U residues at the end of each viral gene is thought to direct polymerase stuttering on the template and result in the addition of poly(A) tails to viral mRNAs. In a separate step in transcription, nontemplated G residues are inserted at a single site within the P/V gene to produce a series of mRNAs, a process referred to as "editing" (Lamb and Kolakofsky, 1996). All nonsegmented negative sense RNA viruses are thought to polyadenylate their mRNAs by polymerase stuttering (Jacques and Kolakofsky, 1991). By contrast, mRNA editing in the P gene appears to be unique to paramyxoviruses, being carried out by all members of this family except RSV and HPIV1. The results presented here support the proposed relationship between paramyxoviruses capable of performing P gene mRNA editing and the preference for replication of 6*N* length genomes (Hausmann *et al.,* 1996).

For SeV, synthetic non-6*N*-length DI genomes that contained the P/V editing site were found to undergo "genome length correction" (Hausmann *et al.,* 1996) by the insertion or deletion of nontemplated bases to produce genomes that were 6*N* in length and these genomes were preferentially replicated. These alterations to genome length occurred at the P/V "slippery site," a *cis*-acting sequence that normally directs the viral polymerase to add nontemplated G residues only during transcription and not replication. SeV genome length correc-

tion by alterations at a polyadenylation site have not been observed (Hausmann *et al.,* 1996). However, the possibility remains that other "slippery" sites might exist within the genome which could direct the viral polymerase to insert or delete nucleotides. In this regard, it was surprising to find that two cloned SV5 DI RNA genomes (DI_{834} and DI_{774}) contained a stretch of five nontemplated A residues inserted precisely at the crossover junction (antigenome-sense; see Fig. 2), and these genomes are of 6*N* length. It is possible that these nontemplated residues were added during the polymerase "departure and reentry" events that are thought to generate copyback RNAs (Perrault, 1981; Holland, 1987). Alternatively, the crossover point of these genomes may have created a unique RNA sequence that directed the addition of nontemplated bases in subsequent rounds of replication. The sequence context within which the nontemplated nucleotides were inserted (3′-UAAAAGAGAAAAACAG; inserted nucleotides underlined) bears little resemblance to either the P/V editing site for SV5 (3′-UUCUCCCC; Thomas *et al.,* 1988) or to the consensus polyadenylation signals found at the end of each coding region (3′-AAANUUCU$_{4-7}$). Regardless of the mechanism, the presence of these nontemplated residues and their resultant restoration of 6*N* genome length argues that "genome length correction," whether by pseudotemplated addition of nucleotides (Hausmann *et al.,* 1996) or by undefined alternative means, may play a role in the successful propagation of naturally occurring non-6*N*-length DI genomes that may be generated during the replication of the paramyxovirus genome.

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