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Modified Model for the Switch from Sendai Virus Transcription to Replication

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RNase mapping was used to estimate the levels of unencapsidated Sendai virus plus-strand RNAs which cross the leader-NP junction relative to NP mRNA. Significant amounts of leader readthrough RNAs were found in Z strain-infected cells, similar to that described for the polR mutant of vesicular stomatitis virus, even though this strain is considered wild type. The levels of the readthrough RNAs detected fell sharply when progressively longer probes were used, unlike that of NP mRNA. These studies suggest that polymerases which read through the first junction terminate shortly afterwards in the absence of concurrent assembly of the nascent chain, whereas those which reinitiate at NP continue efficiently to the next junction. Reinitiation appears to be necessary to convert the polymerase to a mode in which elongation is independent of concurrent assembly. Concurrent assembly appears to be required not only for the polymerase to read through the junction efficiently, but also for it to continue elongation between junctions.

Sendai virus, a member of the *Paramyxoviridae*, contains a nonsegmented minus-strand RNA genome of 15.3 kilobases (kb), from which a leader RNA and six mRNAs are transcribed. The viral polymerase responsible for mRNA synthesis, referred to as the transcriptase, is thought to enter its nucleocapsid (NC) template at or near the 3′ end and to sequentially synthesize the leader and the NP, P/C, M, F, HN, and L mRNAs by terminating and restarting at each of the gene junctions. The mRNAs are capped and polyadenylated during synthesis, whereas the leader RNA is unmodified at either end. The minus-strand genome is also a template for the synthesis of antigenomes, which are unmodified full-length complements of the genome which serve as intermediates in genome replication. The polymerase responsible for antigenome synthesis, which we refer to as the replicase, therefore ignores the junctional stop-start signals that the transcriptase responds to (for a recent review, see reference 16).

The manner in which this switch from transcription to replication takes place for paramyxoviruses, a central event in the life cycle of all minus-strand RNA viruses, is thought to be similar to that proposed for the rhabdovirus vesicular stomatitis virus (VSV) (1, 2, 5, 7, 15). Since, like VSV, Sendai virus genomes and antigenomes are found only encapsidated with the nucleocapsid protein (NP) and their synthesis is dependent on on-going protein synthesis whereas that of mRNA is not, the switch may also be effected here simply by the intracellular concentration of unassembled NP. In this minimal model, the template and the polymerase for mRNA and antigenome synthesis are identical; the difference lies in whether the nascent RNA chain is being concurrently assembled into NCs. Concurrent assembly would somehow be responsible for the polymerase ignoring the junctional signals, all that is required to switch it from a transcriptase into a replicase.

The site for the initiation of NC assembly on VSV leader RNA has been mapped to the first 14 bases at the 5′ end of the chain (7). Polymerases whose nascent leader chains had not assembled with NP before the first junction was reached would terminate and then restart at the beginning of NP to make all the mRNAs. All junctional signals would be observed, as none of the nascent mRNAs could assemble NP. Polymerases whose nascent leader RNAs had begun assembly would not observe any of the junctional signals, because NC assembly would follow the polymerase, resulting in the synthesis of a full-length and simultaneously encapsidated plus-strand RNA. The frequency of genome replication would then be determined by the level of unassembled NP, and the switch would be part of a self-regulatory system controlling the relative amounts of transcription and replication. We refer to this scheme as the minimal model, since the switch is effected entirely by unassembled NP, the only viral protein known to be required for replication that is not also required for transcription.

The organization of the Sendai virus and VSV genomes is also quite similar, including the nature of the junctional signals for transcription (11–13). We would therefore expect that the minimal model would also apply to Sendai virus, at least in broad outline, even though several of the experiments which led to the model have so far only been reported for VSV. Paramyxoviruses such as Sendai virus, however, have an overlapping gene (C) not found in rhabdoviruses, contained within the P gene in an alternate reading frame (12). As the C protein has been localized with NCs intracellularly (4, 20), it may also participate in RNA synthesis. We have therefore further examined Sendai virus plus-strand RNA synthesis to investigate how closely the above model applies.

MATERIALS AND METHODS

Isolation of cytoplasmic RNAs. Confluent cultures of BHK cells in 10-cm dishes were infected with 20 PFU of Sendai virus Harris (H) or Z strain per cell and maintained at 33°C. Cycloheximide was added after adsorption (1 h) at a concentration of 10 μg/ml when indicated. The cells were harvested at various times by scraping into phosphate-buffered saline and recovered by centrifugation. Cytoplasmic extracts were prepared by vortexing 10^7 cells in 0.5 ml of lysis buffer (0.15 M NaCl, 0.05 M Tris hydrochloride [pH 7.4], 0.6% Nonidet P-40 [NP-40], followed by centrifugation for 10 min at 4,000 × *g*. The extract was layered on a preformed 20 to 40% CsCl density gradient and centrifuged for 20 h at 52,000 rpm in an

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SW60 rotor. The RNA pellet was taken up in TNE (25 mM Tris chloride [pH 7.4], 50 mM NaCl, 1 mM EDTA), precipitated in ethanol, and dissolved in ET (1 mM EDTA, 10 mM Tris chloride [pH 7.4]).

In vitro polymerase reaction. Purified Sendai virus (50 μl, 5 mg/ml) grown in LLC-MK2 cells was used in 250-μl reaction mixes containing 50 mM HEPES (*N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.1), 5 mM $MgCl_2$, 5 mM spermidine, 0.05% NP-40, 1 mM each all four NTPs, 100 U of RNasin, 48 mM Na_2HPO_4, and 1 mg of poly-L-glutamic acid per ml. The reaction mix was incubated at 30°C for 3 h and then brought to 0.5 M NaCl and 1% NP40, heated for 2 min at 37°C, and centrifuged on a 20 to 40% CsCl gradient to obtain the pelleted RNAs as above.

RNase protection. Approximately 10^5 cpm of riboprobe were coprecipitated with 1 to 9 μg of CsCl pellet RNA and 20 μg of tRNA, suspended in 9 μl of ET, heated for 1 min at 90°C, quick chilled, and then made 0.3 M NaCl, 20 mM Tris hydrochloride (pH 7.4), 2 mM EDTA (2× buffer A) in a total volume of 10 μl. After annealing for 30 min at 70°C, 50 μl of 0.1% sodium dodecyl sulfate (SDS)–40 μg of RNase A per ml–5 U of RNase T_1 per ml in 2.5× buffer A was added. After 45 min at room temperature, 600 μg of proteinase K per ml and an additional 0.1% SDS were added. After 20 min at 30°C, the volume was raised to 250 μl with TNE and 0.5% SDS, extracted twice with phenol-chloroform, and ethanol precipitated. The remaining RNAs were dissolved in 3 μl of 80% formamide plus dyes, heated for 2 min at 90°C, and separated on a 6% polyacrylamide sequencing gel.

Preparation of riboprobes. The H strain probe (nucleotides [nt] 22 to 142) was derived from pSL3, which contains the 3' end of the genome starting at nt 22 in the *Pst*I site of pBR322 (11). The first site for *Taq*I is at nt 142. This *Pst*I-*Taq*I fragment was inserted into pSP65 at the *Pst*I and *Acc*I sites. After linearization with *Hin*dIII, SP6 polymerase reactions were carried out with 0.5 mM ATP, CTP, and GTP and 50 μCi of [^{32}P]UTP (10). Equal volumes of formamide and dyes were then added, and the radioactive transcript was separated directly on a 6% polyacrylamide sequencing gel. The transcript was localized by autoradiography, and after excision of the band it was eluted by agitating overnight at 4°C in TNE.

The Z strain probes were prepared from clone B36 (21) which contains the 3' end of the genome starting at nt 1 plus 22 extra bases inserted into the *Bam*HI site of pBR322. Three of the riboprobe plasmids containing the entire leader sequence plus variable portions of the NP gene were obtained by *Bam*HI-*Nco*I (nt 358), *Rsa*I (nt 234), and *Taq*I (nt 142) digestion, followed by insertion into the *Bam*HI and *Acc*I/*Hin*cII sites of SP65 (nt 1 to 142) or pGEM3 (nt 1 to 358 and 1 to 234). When the second enzyme was *Nco*I, it was filled in with the Klenow enzyme before *Bam*HI digestion.

The *Sal*I site at nt 58 was created by site-directed mutagenesis in clone 1-142 with *Eco*RI and *Hin*dIII to create the gap and the synthetic oligodeoxynucleotide 3'-AATCCCA GCTGCATAGGTG-5' (the two base changes are underlined). The procedure used was essentially as described before (10). The 81-bp *Bam*HI-*Sal*I fragment containing the first 58 nt of the Sendai virus genome was then inserted into the same sites of pGEM3. All riboprobes were prepared simultaneously with the same labeled and unlabeled nucleotide triphosphates by using DNAs linearized immediately after the inserts to ensure the same specific activity.

The plasmid containing the NP-PC junction (nt 1638 to 1812) was derived from B36 by digestion first with *Acc*I and *Nco*I. The 459-bp fragment was isolated and digested with *Taq*I, and the 174-bp *Nco*I-*Taq*I fragment was isolated, and the ends were blunted with the Klenow enzyme and inserted into the *Sma*I site of pGEM4.

RESULTS

Sendai virus genomes and antigenomes are found only in NC structures, with a buoyant density of 1.31 g/ml in CsCl density gradients (9). When cytoplasmic extracts of infected cells are centrifuged on such gradients, the pellet fraction contains only the unencapsidated viral RNAs, i.e., the mRNAs and some of the leader RNAs (6), without their complements. These pelleted RNAs are suitable for measuring the amounts of the various viral transcripts which cross a gene junction by RNase mapping, since self-annealing of the viral RNAs or annealing of the minus-strand riboprobe with antigenome RNA should not occur.

We first examined the transcripts which cross the leader-NP junction with a minus-strand riboprobe containing nt 1 to 142 (Fig. 1). When similar preparations of CsCl pellet RNA from H strain infections were examined with 3'-end-labeled genome RNA as a probe (17), leader RNAs of 31, 55, and 65 nt were found, the predominant 55-nt species having terminated at the junction. Protected fragments consistent with these species were also weakly seen with the internally labeled riboprobe (Fig. 2, marked l), which detects NP mRNA (87-nt band) and leader-NP readthrough transcripts (142-nt band) as well. (In this experiment, a small amount of the intact 240-nt riboprobe, of which only the central 142 nt are virus specific, survived digestion in all cases). The origin of these bands is defined by their expected lengths, by their ability to anneal to riboprobes specific to either the leader or NP mRNA regions when the gel is converted into a Northern blot (not shown), and whether their mobility is unchanged when a nested set of riboprobes is used (Fig. 3A). The leader RNAs are minor species compared with the NP mRNA in these samples, possibly because they are less stable or have been encapsidated (6) and are removed during preparation. The most striking result, however, is the relative amount of leader-NP readthrough transcripts.

The 142-nt band presumably results from a transcript which initiated at nt 1 and extended to at least nt 142 and was not encapsidated, as judged by CsCl density gradient centrifugation. These transcripts are not antigenomes which had somehow escaped the separation protocol, as riboprobes from the opposite end of the genome could not detect any plus-strand RNA other than 3' end of the L mRNA, even though the L mRNA was 20-fold less abundant than NP mRNA (not shown). We also examined the relative amounts of these transcripts during the time course of the infection. RNA was prepared from cells harvested at 4 h as well as 18 h postinfection and at 4 h from cultures incubated in the presence of cycloheximide. The inhibition of protein synthesis prevented genome replication but not transcription, so that under these conditions the viral RNAs were predominantly the result of primary transcription. When all these samples were compared (Fig. 2) and the results were quantitated by densitometry and normalized for the number of uridines in each fragment, the readthrough RNA represented 39% of the NP mRNA at 4 h when new genome synthesis was prevented with cycloheximide, 43% at 4 h without drug, and 19% at 18 h. Unencapsidated leader readthrough RNAs are thus present at relatively high levels relative to NP mRNA throughout the infection.

The readthrough transcripts appeared to be too abundant and stable to be true replication intermediates, which are

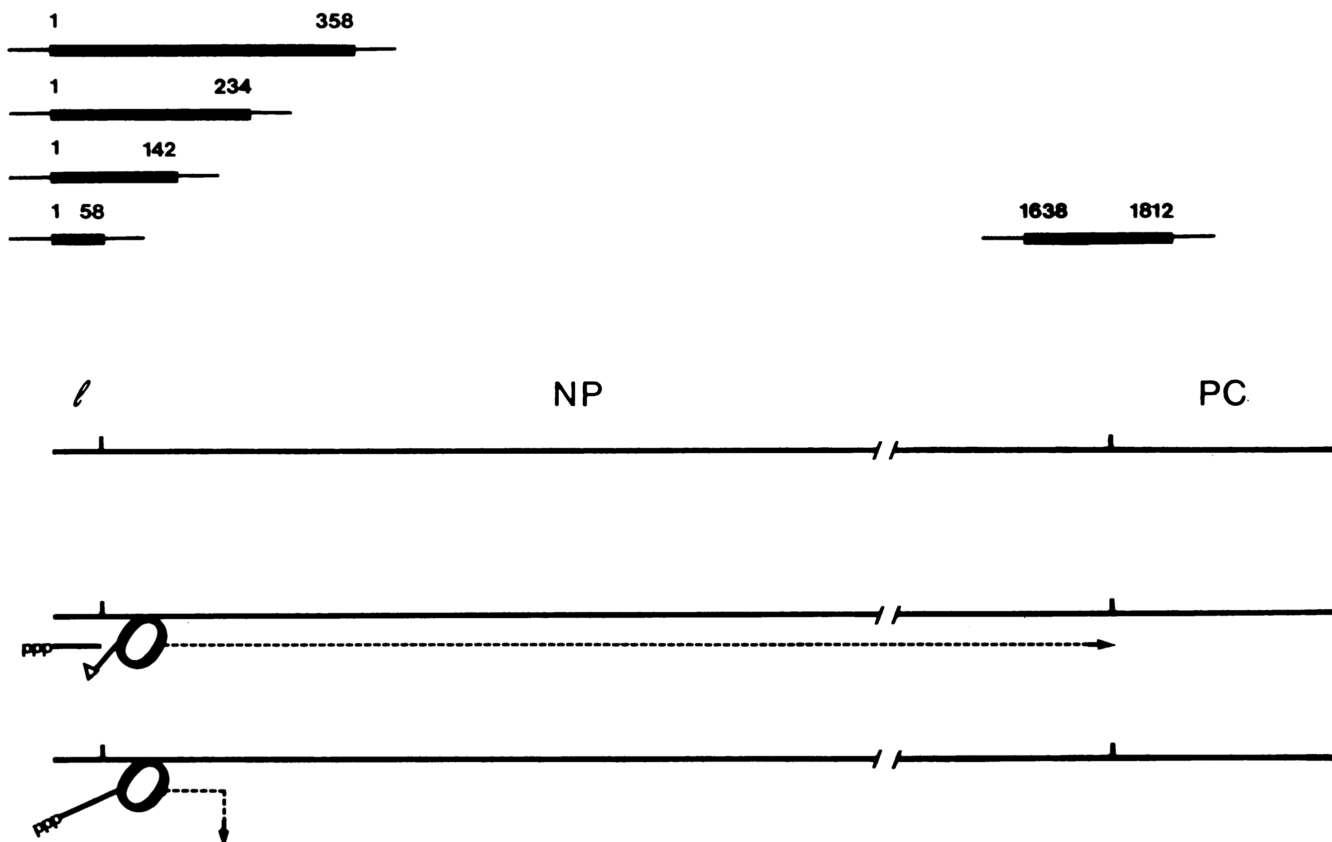


FIG. 1. Schematic diagram of riboprobes used and the modified model. The middle line represents the 3′ or left end of the minus-strand genome with the leader, NP, and PC regions separated by vertical bars representing the junctions. Map positions are numbered from this end or the 5′ end of the antigenome. Above are shown the Z strain riboprobes used. The extents of the viral sequences are indicated by heavy lines and map positions; the thin lines at the ends indicate transcribed vector sequences. Below are shown the two situations of plus-strand RNA synthesis found in Z strain-infected cells in the absence of concurrent assembly. In each case the polymerase is shown just after having crossed the first junction, but with or without having reinitiated, and the consequences for continued elongation.

expected to be very transient species. Their levels relative to NP mRNA were reduced by only twofold between 4 and 18 h, whereas NP mRNAs increased by more than 30-fold between these times. Bona fide replication intermediates described for VSV (14) were found to represent only a small fraction of the RNA made in a 5-min pulse of [^{3}H]uridine and were difficult to see at all after longer labeling. They were also considerably larger (2,000 to 11,000 nt) and, more importantly, were assembled with N protein, as their RNAs were resistant to RNase. The pelleted Sendai virus readthrough transcripts, on the other hand, were only a few hundred nucleotides in length, sensitive to RNase (not shown), and made at the same rate relative to NP mRNA in the absence of viral protein synthesis. They therefore do not require assembly with NP for their synthesis either during primary transcription in vivo or in vitro (see below). True Sendai virus replication intermediates must of course also be present in these infections, but they would be expected to be retained in the CsCl gradient, along with mature genomes and antigenomes.

Polymerases which read through the leader-NP junction. This level of leader readthrough RNAs was unexpected, as nascent chains which had not begun assembling with NP should have terminated efficiently at the first junction. It was therefore of interest to characterize further the polymerases which made readthrough RNAs; e.g., did they continue along the template until the next junction, which might now be observed, or did they somehow sense the absence of NC assembly on the nascent chain and abort prematurely? For this experiment, a nested set of minus-strand riboprobes containing nt 1 to 58, 142, 234, and 358 were used (Fig. 1). The mappings were again carried out at more than one RNA concentration to ensure that the results were proportional, as for Fig. 2. The gel from one such experiment is shown in Fig. 3A. Riboprobe 1-58 only detected the leader region plus 3 nt, but the other three detected both NP mRNA and readthrough RNAs. Besides riboprobe 1-142, described before, riboprobes 1-234 and 1-358 detected protected fragments of 180 and 302 nt (NP mRNA) and 234 and 358 nt (leader-NP readthrough), respectively. In this experiment, the bands were cut out and counted, and the results were plotted as counts per uridine per minute in each fragment, so that the relative amounts of each species could be compared directly (Fig. 3B). Except for riboprobe 1-358, a straight line could be drawn through the origin with at least two of the three points, and tripling the protecting RNA at each step led to three times as many counts protected. As the relative amounts of the various RNAs are given by the slopes of the lines, it is clear that there is a decreasing abundance of readthrough RNAs as a function of how far their 3′ ends had extended. This can also be seen in Fig. 3C, in which the various readthrough RNAs from the lowest concentration point used for all four probes (from three separate experiments) are plotted as the percentage of the NP mRNA

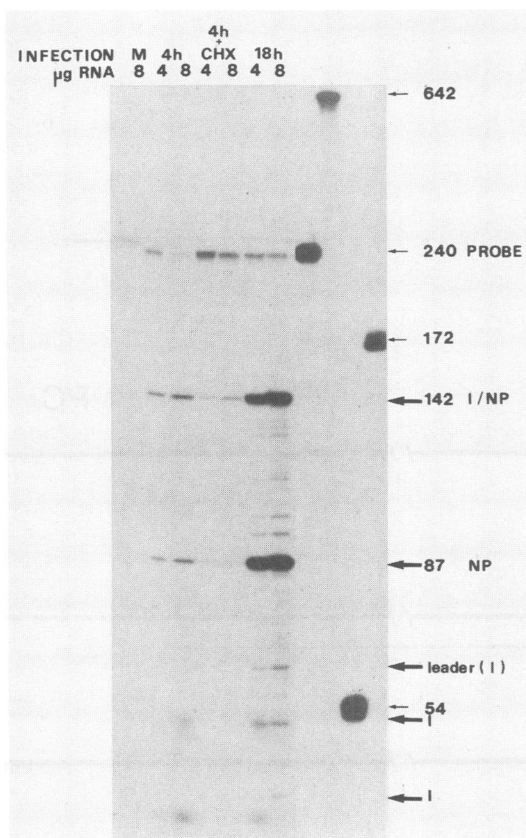


FIG. 2. Detection of Sendai virus plus-strand RNAs in CsCl pellet RNA by riboprobe 1-142. CsCl pellet RNA from mock-infected (M) or virus-infected cells harvested at 4 h with or without cycloheximide (CHX) added after virus adsorption or 18 h were assayed for plus-strand RNAs with a minus-strand riboprobe containing positions nt 1 to 142. The amount of the protecting RNA used in each lane is indicated above. The four right-hand lanes show riboprobes run as length markers (small arrows). The large arrows show the viral RNAs detected. Three leader RNAs are indicated by the letter l.

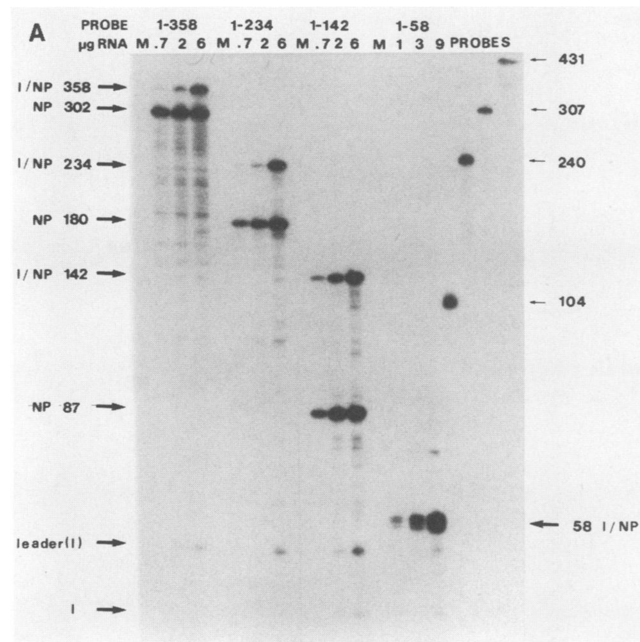
detected by the same probe. There was no tendency for RNAs which had initiated at nt 56 (NP mRNA) to decrease in abundance, as measured by probes which extend progressively downstream, whereas the readthrough RNAs decreased from 19% of NP mRNA at nt 58 to 3% at nt 358. Furthermore, less than 10% of the polymerases which started the NP mRNA failed to finish it (Fig. 4A), whereas we have been unable to detect any complete leader-NP readthrough RNAs by Northern analysis with leader-specific riboprobes (not shown).

In these experiments, the mappings were carried out with more than one concentration of protecting RNA to ensure that the riboprobe was not limiting for two reasons. First, under conditions of limiting riboprobe, formation of the longer hybrids might occur preferentially. More importantly, under these conditions a riboprobe might be simultaneously hybridized to both a leader and an NP mRNA without any gap in between, as leader RNA may extend to nt 55 and NP begins at nt 56. Such a composite hybrid might not be sensitive to RNase attack and appear as a readthrough transcript in this assay. The more the riboprobe is in excess, however, the less likely it is that a composite hybrid would form. As shown in Fig. 3, the ratio of readthrough RNA to NP mRNA did not change even when nine times less protecting RNA from a given sample was used, and we measured more rather than less readthrough RNA relative to NP mRNA when there was 30 times less viral RNA in the sample at 4 h relative to 18 h postinfection (Fig. 2). These results are inconsistent with the measurement of composite hybrids, as is the finding that readthrough RNAs become less abundant relative to NP mRNA when progressively longer riboprobes are used. Finally, as the sequence on the riboprobe preceding the junction is AAAA, in some experiments we used RNase T_2 instead of RNases A and T_1, but this did not change the results (not shown). As the readthrough RNAs do not appear to be artifacts of the method, these results indicate that polymerases which start at nt 1 and readthrough the first junction terminate heterogeneously shortly thereafter, whereas those which reinitiate at NP continue at high frequency to the next junction.

Z and H strains of Sendai virus. Several strains of Sendai virus are available, and the one we have described so far is the Z strain, the only one which has been cloned completely (21, 22). The sequence of the H strain with which we have worked in the past has been cloned up to the L gene, but our clone closest to the left end starts at nt 22 (11). However, as these strains are 99% conserved at the nucleotide level, the Z strain riboprobes should also be able to measure the H strain transcripts. When equal amounts of CsCl pellet RNA from Z and H strain-infected cells were used to protect the Z strain riboprobe 1-142, these samples were found to contain similar amounts of NP mRNA but very different amounts of leader readthrough RNAs (Fig. 4A). By direct counting as for Fig. 3, the Z strain sample was found to contain 12.2% as much readthrough RNAs as NP mRNAs, in good agreement with the 14% average in Fig. 3C, but the H strain sample was found to contain 20 times less (0.6%). To ensure that this difference was not due to the slight differences in sequence between the two strains in this region, the amount of readthrough RNA in H strain CsCl pellet RNA was measured with an H strain riboprobe containing nt 22 to 142. As shown in Fig. 4B, this homologous probe again detected less than 1% readthrough RNA in the in vivo sample relative to NP mRNA under conditions in which the products of an H virion polymerase reaction were found to contain considerable amounts of readthrough RNA. The ability of the Z strain polymerase to readthrough the first junction at an unexpectedly high frequency in vivo is thus not shared by the H strain.

Since the Z and H strain polymerases readthrough the first junction at such different frequencies, we examined the readthrough frequency at the next junction (NP-PC), by using a Z strain riboprobe containing positions nt 1638 to 1812 (the NP mRNA ends at nt 1736, and PC begins at nt 1740). As shown in Fig. 4A, this probe detected roughly equal amounts of NP (100 nt) and PC (71 nt) mRNAs, as well as NP-PC readthrough RNAs (174 nt) in equal amounts of Z and H CsCl pellet RNA. However, the level of readthrough at this junction by the Z and H polymerases was identical to that of the H polymerase at the first junction (0.6 and 0.5%, respectively). The Z strain polymerase therefore reads through the first and second junctions at different frequencies, whereas the H strain polymerase reads through both at the same low frequency.

In vitro studies. The level of transcripts from the leader-NP region that we measured in vivo are steady-state levels, determined by turnover as well as synthesis. Moreover, for transcripts which contain the leader sequences, turnover in this context includes encapsidation as well as degradation.



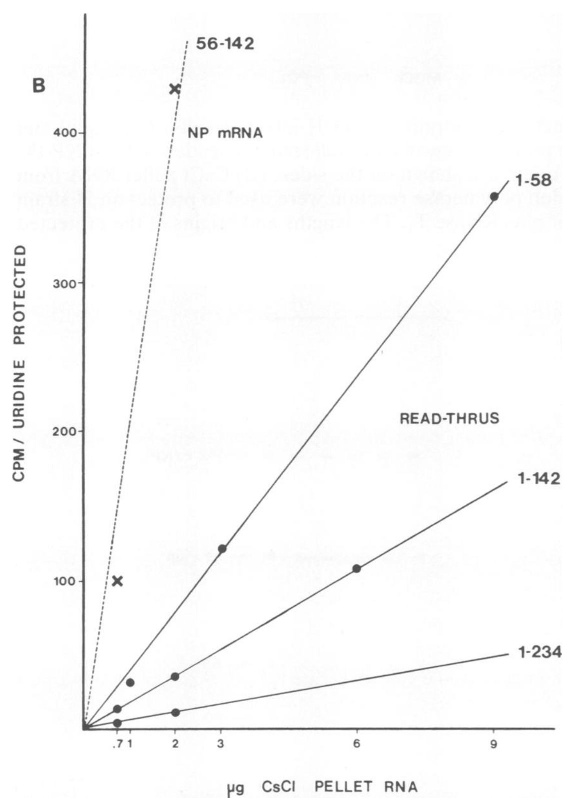
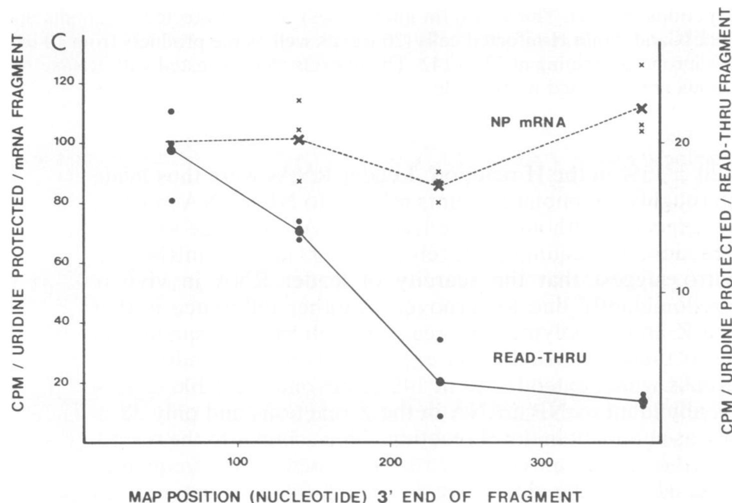


FIG. 3. Quantitation of leader readthrough and NP mRNAs with a nested set of riboprobes. (A) Three different amounts of CsCl pellet RNA (18 h) each a threefold increase, were used to protect riboprobes containing positions 1 to 58, 142, 234, and 358 (Fig. 1), and the RNAs remaining after digestion were separated by electrophoresis. The autoradiogram from one such experiment is shown; the lengths (in nucleotides) of the various probes used and RNAs detected are indicated on the sides. The probes used in each experiment are indicated above, as is the amount of protecting RNA for each lane. M refers to 6 or 9 µg of RNA from mock-infected cells. The first eight lanes containing the larger probes were exposed for shorter times than those containing the smaller probes. (B) Bands representing the leader readthrough RNAs (l/NP) and the NP mRNAs (NP) were excised from a parallel gel which contained blank lanes in between, and their Cerenkov radiation was determined in a beta counter. After background subtraction (30 cpm total), the counts present in each band were divided by the number of uridines and plotted as a function of the amount of protecting RNA for the three smallest riboprobes. Only the NP mRNA bands from riboprobe 1-142 are shown. (C) Results obtained with the lowest amount of protecting RNA from three separate experiments, one of which is shown in panels A and B, are plotted according to the 3' end of the plus-strand fragment protected in the various hybrids. To normalize the values for the NP mRNA, the average of the values from the three riboprobes in each experiment (115, 34, and 189 cpm/U protected) was set to 100, and the results from each riboprobe are plotted relative to this value. The readthrough RNAs, on the other hand, are plotted relative to the NP mRNA detected by the same probe in the same experiment, except for that detected by riboprobe 1-58, which is plotted relative to the average value for the NP mRNA. Large and small crosses and circles indicate the mean and individual values for NP mRNA and the leader readthrough RNAs, respectively.



We therefore also examined readthrough in vitro with purified Sendai virions, as degradation of the uncapped RNAs containing the leader region might be less important here than in vivo, and encapsidation of these transcripts should not take place. RNA levels may therefore more closely approximate their rates of synthesis in vitro. Polymerase reaction mixes containing equal amounts of Z and H strain virions were made, and the products were examined with the Z strain riboprobe 1-142 as before. To distinguish between RNA made de novo and that present as virion contaminants, mock reaction mixes containing excess EDTA were processed in parallel. As shown in Fig. 5, the results in vitro were basically similar to those in vivo, but there were some notable differences. The leader RNAs, which were very minor species in vivo, were more prominent in vitro. Z strain virions made two leader RNAs which migrated at approximately 55 and 40 nt in length, and H virions made an additional leader band at 25 nt (which was also present in vivo [not shown]). When the results were quantitated by densitometry and normalized for uridine content and the signal due to contaminating NP mRNA (EDTA reaction) was subtracted, there was 62 ± 4% as much leader as NP mRNA in the Z reaction when the leader bands were summed, and

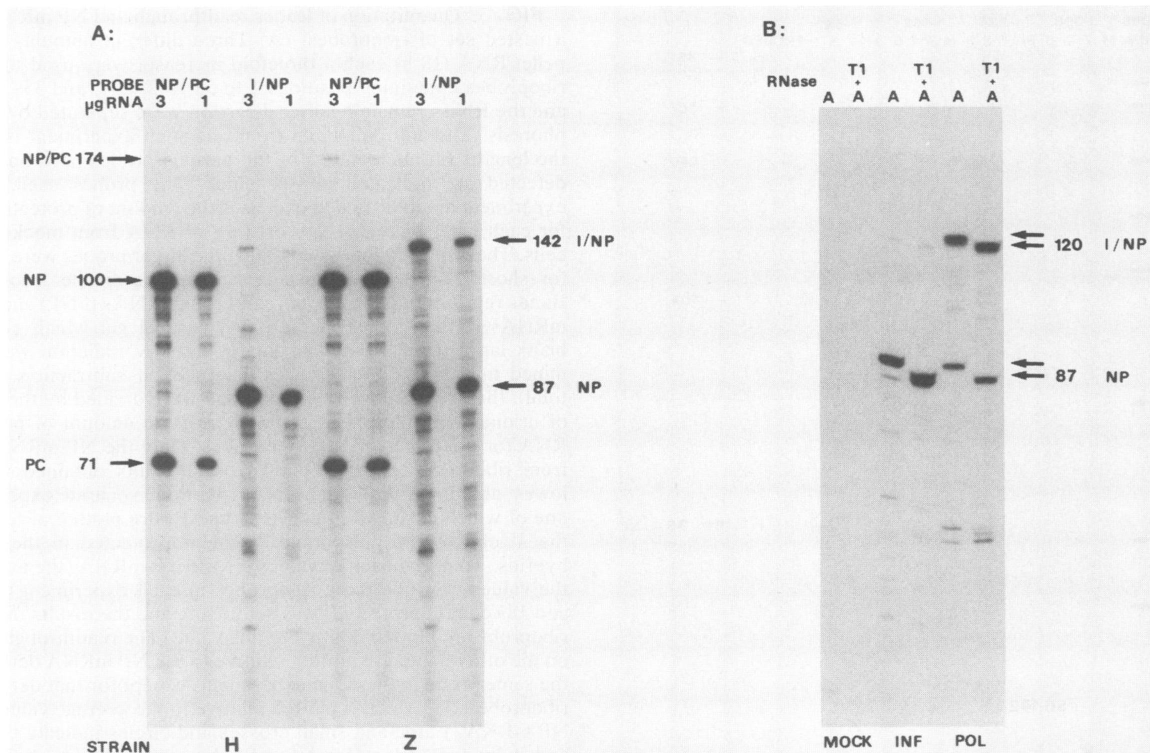


FIG. 4. Estimation of readthrough frequency at the leader-NP and NP-PC junctions in strain Z- and H-infected cells. (A) Equal and increasing amounts of CsCl pellet RNA from strain Z and H infections were used to protect riboprobes which span the leader-NP and NP-PC junctions (Fig. 1). The length (in nucleotides) of the protected fragments and their origin are shown on the sides. (B) CsCl pellet RNA from mock- and strain H-infected cells (20 μg) as well as the products from 10 μl of a virion polymerase reaction were used to protect an H strain riboprobe containing nt 22 to 142. The hybrids were digested with RNase A alone or plus RNase T_1. The lengths and origins of the protected bands are indicated on the side.

120 ± 13% in the H reaction. Leader RNAs were thus made in roughly equimolar amounts relative to NP mRNA in vitro, as expected, although more leader RNA was made in the H reaction. The equimolar levels of leaders and NP mRNAs in vitro suggest that the scarcity of leader RNA in vivo is predominantly due to turnover. Another difference is that the Z and H polymerases read through more frequently in vitro than in vivo. In this experiment, leader readthrough RNAs which extended to nt 142 or beyond were 106 ± 12% as abundant as NP mRNA in the Z reactions and only 38 ± 4% as abundant in the H reaction. Thus, similar to the results in vivo, the Z polymerase reads through more frequently than H, but the difference is only 3-fold, as opposed to 20-fold in vivo. In addition, similar results were obtained in vitro when the reaction products were radioactive and the riboprobe was not, and here the readthrough RNAs cannot be the result of composite hybrids.

The apparent difference in readthrough frequency in vivo and in vitro of both strains is unlikely to be due entirely to more turnover in vivo, as we measured only 39% readthrough with the Z strain in vivo at 4 h even when protein synthesis was inhibited to prevent encapsidation. Furthermore it is difficult to accept that the H strain difference of 63-fold (0.6 versus 38%) is only a question of turnover. It appears more likely that this difference reflects the peculiar conditions of the in vitro reaction. For example, the virion reactions take place within envelopes containing the M protein, which may be a transcriptional inhibitor. Furthermore, the optimum conditions for the in vitro reaction are

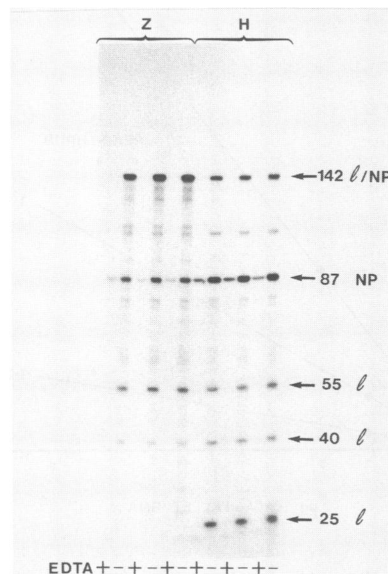


FIG. 5. Polymerase readthrough in Z and H virion reactions. Triplicate nonradioactive in vitro polymerase reactions were carried out with both strains as described in Materials and Methods. Mock reactions were also done in parallel, which contained an additional 20 mM EDTA as indicated, to prevent de novo synthesis. The products from 10 μl of each reaction were analyzed by RNase mapping with riboprobe 1-142. The lengths of the leader RNAs (script l) were estimated relative to those of riboprobes of known length (not shown) and are therefore approximate.

quite different from those of VSV. For Sendai virus, the replacement of Tris with HEPES buffer, chloride or acetate with phosphate as counter ions, and the addition of poly-L-glutamic acid, led to a 500-fold stimulation of activity. All or any of the above could account for the differences between readthrough in vivo and in vitro. We also note that whereas parallel reactions were remarkably reproducible (Fig. 5), readthrough levels varied significantly from one set of reactions to the next, even with the same preparation of virus, for reasons that are unclear (not shown).

DISCUSSION

RNase mapping is well suited to examine transcripts which cross gene junctions, as all three possible transcripts can be measured simultaneously with a single probe. However, as it is unreliable when the complementary RNA is also present and leader readthrough RNAs cannot be distinguished from antigenomes by this method, we have restricted ourselves to characterizing the CsCl pellet RNAs. We therefore do not know what fraction of the leader readthrough RNAs are represented by this material. However, by comparing their levels to those of the NP mRNA, which quantitatively pellets in CsCl gradients and is thought to be the most stable of these RNAs, we can get a minimum estimate of their rates of synthesis. The time course of their accumulation during Z strain infections suggests that their levels at 18 h, when most of our measurements were made, do in fact underestimate their rate of synthesis relative to NP mRNA, but only by about twofold.

We were surprised to find such large amounts of leader readthrough RNAs in Z-infected cells which had not been encapsidated but not in H strain infections. The presence of easily measurable amounts of the readthrough RNAs allowed us to measure how far their 3′ ends had extended. According to the minimal model, polymerases which read through the first junction would be expected to continue on to the next. Concurrent assembly is supposedly only required for the polymerase to disregard the junctional signals and should have no effect between junctions. This test of the model has clearly not held up for the Z strain, as these replicases aborted synthesis shortly after having crossed the first junction, even though they are traversing the same template sequences as the transcriptases. Z strain transcriptases and replicases can thus be distinguished even in the absence of concurrent assembly.

Very few, if any, of the polymerases which read through the first junction continue on to the next. Readthrough of the second junction can then only take place with polymerases which had reinitiated at the first, i.e., transcriptases. As the polymerases of the Z and H strains are indistinguishable by their frequency of readthrough at the second junction (0.6 and 0.5%, respectively) but vary 20-fold in the frequency with which they read through the first junction, this again suggests that these polymerases must somehow be different, independent of assembly.

The reason for the difference in these readthrough frequencies at the first junction remains unknown. However, a remarkably similar situation has previously been described for VSV. Perrault and co-workers, using a selection protocol for heat resistance, isolated mutants (polR) with enhanced ability to read through the leader-N junction but not subsequent junctions (18, 19). The enhanced ability to read through the junction is reproducible in vitro, and here it maps to the N protein of the template rather than the exchangeable polymerase components (L and NS). The VSV polR mutants stress the point that the assembled N or NP protein, which is an integral part of the template, is thus an integral part of the polymerase complex as well. The differences between the Z and H polymerases could then also lie in the template component of the complex, but any component of this complex could of course be equally responsible for the readthrough phenotype. This might now also include the Sendai virus equivalent of the simian virus 5 and measles virus V proteins (23; R. Cattaneo, K. Kaelin, and K. Baczko, Cell, in press), which does in fact exist as predicted (unpublished).

More importantly, what differences between the replicase and transcriptase can account for the finding that the former requires concurrent assembly to continue down the template when it has crossed the first junction, whereas the latter does not? And to what extent does the minimal model still apply to Sendai virus? A key element of this model is that the switch from transcription to replication is controlled not at the level of chain initiation, but during elongation. This would also appear to be true for Sendai virus, as leader readthrough RNA synthesis continues similarly to NP mRNA synthesis in the presence of cycloheximide (Fig. 2). In that case, transcriptases and replicases cannot be distinguished until the polymerase has crossed the first junction. It would appear that the act of crossing the junction is responsible for this difference. Before the first junction, only one form of the polymerase can be distinguished, that which has initiated at nt 1. After this junction there are two forms; transcriptases which have reinitiated at NP and whose nascent chains no longer carry the leader sequences and are capped, and replicases which have not reinitiated and whose nascent chains still contain the leader, with a 5′ triphosphate. Any or all of these may account for the difference in how they continue elongation without assembly.

Exchange or modification of polymerase subunits during reinitiation at the junction may also be involved. In the case of VSV polymerase reactions in vitro (3, 19), wild-type (wt) N mRNA synthesis requires high ATP concentrations for half-maximal synthesis, whereas leader synthesis takes place efficiently at lower ATP concentrations. In the polR mutants, however, N mRNA synthesis behaves similarly to leader synthesis with respect to ATP. These results are consistent with different phosphorylation states of the polymerases which initiate leader and N mRNA synthesis, and these differences could take place during reinitiation at the first junction. In the case of paramyxoviruses, it has been found that in a persistent measles virus infection of human brain, a single gene (*M*) could be highly and specifically mutated whereas these mutations stopped precisely at the flanking PC junction (R. Cattaneo, A. Schmid, K. Baczko, V. ter Meulen, and M. A. Billeter, Cell, in press). One explanation they advance is that during replication a highly mutagenic polymerase copied the *M* but not the flanking genes. This suggests that polymerases could also be exchanged at gene junctions during replication.

The minimal model clearly requires some modification to account for the Z strain infections. A modified model which more adequately describes both Sendai virus Z and H as well as VSV polR and wt is one in which the polymerase, in the absence of concurrent assembly, cannot continue for very long on its template unless it has reinitiated. How frequently it would stop at or read through the junction without assembly could of course be controlled genetically. Concurrent assembly would be required here not only for the polymerase to read through junctions, but also for it to read through continuously. The leader-NP junction would only be

the first of many places where the polymerase would terminate without assembly if it had not reinitiated. The intracellular level of unassembled NP would still be the key element in the switch and would remain the rate-determining one. It would still act by initiating NC assembly on the nascent leader sequences, thereby controlling the rate with which polymerases continue without interruption to the end of the template. Initiation of nascent-chain assembly before the polymerase had reached the junction would result in efficient readthrough regardless of the basal rate of readthrough in the absence of assembly. It should be noted that our data do not rule out the possibility that the polymerases (and/or templates) are committed to either the transcription or replication mode before initiation, but these differences only become apparent at the first junction. This possibility appears less likely, however, as virions would be expected to contain predominantly those in the transcription mode, whereas in vitro they read through the first junction more frequently than is found in vivo (Fig. 5).

Finally, in Z versus H strain infections, termination at the first junction is relatively inefficient, yet this appears to have only a modest effect, as the Z strain grows only a little less quickly than H (again similar to polR and wt VSV). As a majority of the polymerases which read through terminate within 100 nt, some of them may move back to the junction and reinitiate, as suggested for the respiratory syncytial virus polymerase at the 22K-L junction (8). However, the modest effect seen in one-step growth curves in culture could of course have been important in the natural evolution of this strain, which is considered to be as wt as the H strain.

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LITERATURE CITED

1. **Arnheiter, H., N. L. Davis, G. Wertz, M. Schubert, and R. A. Lazzarini.** 1985. Role of nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. Cell **41**:259–267.
2. **Banerjee, A. K.** 1987. Transcription and replication of rhabdoviruses. Microbiol. Rev. **51**:66–87.
3. **Beckes, J. D., A. A. Haller, and J. Perrault.** 1987. Differential effect of ATP concentration on synthesis of vesicular stomatitis virus leader RNAs and mRNAs. J. Virol. **61**:3470–3478.
4. **Bellini, W. J., G. Englund, S. Rozenblatt, H. Arnheiter, and C. D. Richardson.** 1985. Measles virus P gene codes for two proteins. J. Virol. **53**:908–919.
5. **Blumberg, B. M., M. Leppert, and D. Kolakofsky.** 1981. Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. Cell **23**:837–845.
6. **Blumberg, B. M., and D. Kolakofsky.** 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. J. Virol. **40**:568–576.
7. **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.
8. **Collins, P. L., R. A. Olmsted, M. K. Spriggs, P. R. Johnson, and A. J. Buckler-White.** 1987. Gene overlap and site-specific attenuation of transcription of the viral polymerase L gene of human respiratory syncytial virus. Proc. Natl. Acad. Sci. USA **84**:5134–5138.
9. **Compans, R. W., and P. W. Choppin.** 1967. Isolation and properties of the helical nucleocapsid of the parainfluenza virus SV5. Proc. Natl. Acad. Sci. USA **57**:949–956.
10. **Curran, J. A., and D. Kolakofsky.** 1988. Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. EMBO J. **7**:245–251.
11. **Dowling, P. C., C. Giorgi, L. Roux, L. A. Dethlefsen, M. E. Galantowicz, B. M. Blumberg, and D. Kolakofsky.** 1983. Molecular cloning of the 3′-proximal third of Sendai virus genome. Proc. Natl. Acad. Sci. USA **80**:5213–5216.
12. **Giorgi, C., B. M. Blumberg, and D. Kolakofsky.** 1983. Sendai virus contains overlapping genes expressed from a single mRNA. Cell **35**:829–836.
13. **Gupta, K. C., and D. W. Kingsbury.** 1984. Complete sequences of the intergenic and mRNA start signals in the Sendai virus genome: homologies with the genome of vesicular stomatitis virus. Nucleic Acids Res. **12**:3829–3841.
14. **Hill, V. M., C. C. Simonsen, and D. F. Summers.** 1979. Characterization of vesicular stomatitis virus replicating complexes isolated in renografin gradients. Virology **99**:75–83.
15. **Kolakofsky, D., and B. M. Blumberg.** 1982. A model for the control of non-segmented negative strand viruses genome replication, p. 203–213. *In* B. W. J. Mahy, A. C. Minson, and G. K. Darby (ed.), Virus Persistence Symposium 33. Society for General Microbiology Ltd., Cambridge University Press, Cambridge.
16. **Kolakofsky, D., and L. Roux.** 1987. The molecular biology of paramyxoviruses, p. 277–279. *In* P. Bercoff (ed.), The molecular basis of viral replication. Plenum Publishing Corp., New York.
17. **Leppert, M., L. Rittenhouse, J. Perrault, D. F. Summers, and D. Kolakofsky.** 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell **18**:735–747.
18. **Perrault, J., G. M. Clinton, and M. A. McClure.** 1983. RNP template of vesicular stomatitis virus regulates transcription and replication functions. Cell **35**:175–185.
19. **Perrault, J., and P. W. McClear.** 1984. ATP dependence of vesicular stomatitis virus transcription initiation and modulation by mutation in the nucleocapsid protein. J. Virol. **51**:635–642.
20. **Portner, A., K. C. Gupta, J. M. Seyer, E. H. Beachey, and D. W. Kingsbury.** 1986. Localization and characterization of Sendai virus nonstructural C and C′ proteins by antibodies against synthetic peptides. Virus Res. **6**:109–121.
21. **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 sequence of viral NP, P and C proteins. Nucleic Acids Res. **11**:7317–7338.
22. **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.
23. **Thomas, S. M., R. A. Lamb, and R. G. Paterson.** 1988. Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. Cell **54**:891–902.