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# Initiation of Vesicular Stomatitis Virus Mutant polR1 Transcription Internally at the N Gene In Vitro

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**The vesicular stomatitis virus (VSV) polymerase is thought to initiate transcription of its genome by first copying a small leader RNA complementary to the 3′ end of the template. The polR VSV mutants, in contrast to wild-type virus, frequently read through the leader termination site during transcription in vitro. To shed light on polymerase termination and reinitiation events at the crucial leader-N gene junction, we employed RNase protection assays to precisely measure molar ratios of leader, N, and readthrough transcript accumulation in vitro. Wild-type virus synthesized essentially equimolar amounts of leader and N transcripts, but, unexpectedly, the polR1 mutant yielded about twice as much N mRNA as leader (ratio of 1.9 ± 0.1). Primer extension assays ruled out an increase in abortive N transcript synthesis for polR1. Transcription entailed multiple rounds of synthesis, with transcript ratios remaining the same after 0.5 or 2 h of synthesis, ruling out a significant contribution from polymerases "pre-positioned" at the N gene. No significant degradation of either leader or N transcripts was observed after incubating purified products with virions. Our data lead us to conclude that transcription can initiate internally at the N gene, at least in the case of polR1 VSV. We propose, however, that productive internal initiation of transcription is a fundamental property of the VSV polymerase and that of related viruses. A model postulating two distinct polymerase complexes, one for leader synthesis and one for internal initiation, is presented.**

Our understanding of viral genome transcription and replication in the order *Mononegavirales* (nonsegmented negative-strand RNA viruses) draws extensively from earlier studies with vesicular stomatitis virus (VSV), the prototype of the *Rhabdoviridae* family (for reviews, see references 1, 10, 22, and 38). The current model of VSV genome transcription postulates that the viral polymerase complex (P and L proteins) transcribes the viral genome in a sequential stop-start fashion, beginning at the 3′ end of the template (N-RNA complex). The first transcript synthesized is a nonpolyadenylated leader RNA (47 nucleotides [nt]), followed by synthesis of mRNAs encoding N, P/C, M, G, and L proteins. Conserved sequences at the starts and ends of genes provide the signals for termination, polyadenylation, and reinitiation (30). Transcript accumulation also decreases as a function of distance from the 3′ end promoter, apparently due to an inherent tendency for the polymerase to dissociate from the template while pausing at gene junctions (19). In contrast to transcription, replication requires concurrent encapsidation of nascent chains by the P-N assembly complex. The assembly process somehow causes the polymerase to ignore gene junctions. The full-size encapsidated antigenome, in turn, serves as template for the coupled replication and assembly of progeny genomes.

Sequential synthesis of VSV mRNAs is firmly established from UV inactivation and kinetic data, but evidence that leader is synthesized first is more indirect and rests, for the most part, on in vitro transcription reconstitution studies under partial-reaction conditions (9). Concomitant synthesis of leader and mRNA start sequences was observed with detergent-disrupted virions, in agreement with previous observations (35), but only leader starts were produced by the addition of soluble polymerase to purified templates. Both types of oligonucleotides appeared, however, if reconstituted complexes were first preincubated under complete-reaction conditions. Emerson (9) thus proposed that soluble polymerase complexes always initiate synthesis at the 3′ end of the template but that active complexes also become trapped at internal gene junctions during packaging. A subsequent study, however, also employing reconstituted polymerase complexes, found that both types of start sequences, as well as oligonucleotides of unknown origin, are synthesized under partial-reaction conditions, suggesting that such conditions may not reflect bona fide transcription (36).

The 3′ entry model requires that leader RNA be transcribed at least as frequently as N mRNA. Reliable quantitation of leader synthesis has so far been obtained solely from in vitro studies because this small transcript is apparently unstable in infected cells (5, 17, 37). For VSV, a leader-to-N-transcript molar ratio of ~7.0 was obtained by using transcription reconstitution conditions with optimal amounts of L and P proteins (8) and a ratio of ~2.9 was obtained by using detergent-disrupted virions (11). For Sendai virus, roughly equimolar synthesis was found in one case (37) and a large excess of leader over N transcript (ratio, ≥64) was found in the other (23). The wide range in measured ratios is perhaps not surprising in light of several studies showing preferential inhibition of mRNA synthesis relative to leader synthesis under a variety of reaction conditions, including low ATP concentration (2), replacement of ATP with the nonhydrolyzable analog β,γ-imido ATP (13, 26), suboptimal amounts of P protein (8), and addition of a monoclonal antibody reactive with both P and N proteins (39). The presence of aurintricarboxylic acid or vanadyl ribonucleoside complexes, on the other hand, reportedly inhibited all VSV RNA synthesis in vitro, except for synthesis of a capped 68-nt-long N gene transcript (34). This peculiar phenomenon led the authors to suggest the existence of two polymerase complexes, one for leader synthesis and one for mRNA synthesis.

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We reported some years ago that the VSV polR mutants read through the leader-N gene junction at high frequency in vitro, despite the absence of an assembly complex (24). Although mutant readthrough transcripts terminate heterogeneously within the N gene, polR viruses also synthesize properly terminated leader and mRNAs, the latter containing slightly longer poly(A) tails (25). Curiously, high ATP concentrations are not required for mRNA synthesis by polR virus, in contrast to synthesis by wild-type virus (2), and the mutants also tolerate replacement of ATP with β,γ-imido ATP (26). The unusual polR phenotype has been traced to a single amino acid substitution in the template-associated N protein (6, 15, 24). A similar assembly-independent readthrough of the leader-N gene junction has also been described for Sendai virus. In this case, the Z virus strain appears to behave like polR VSV and the H strain appears to behave like wild-type VSV (20, 37).

To clarify the changes in polR mutant transcription in vitro, we measured in this study rates of synthesis of leader, N, and readthrough transcripts by wild-type and mutant viruses under optimal conditions in vitro. Wild-type virus synthesized equimolar amounts of these transcripts, but, unexpectedly, polR1 produced a nearly twofold molar excess of N transcripts. Our findings indicate that N mRNA synthesis can initiate independently of leader synthesis in polR and lead us to question the current stop-start model of transcription in nonsegmented negative-strand RNA viruses.

(The findings documented here were presented in part at the 9th International Conference on Negative Strand Viruses, Estoril, Portugal, 2 to 7 October 1994; at the 14th Annual Meeting of the American Society for Virology, University of Texas at Austin, Austin, Tex., 8 to 12 July 1995; and at the Symposium Honoring John J. Holland, Laguna Beach, Calif., 2 to 4 November 1995.)

## MATERIALS AND METHODS

**Growth and purification of viruses.** Wild-type and polR1 mutant VSVs (Indiana serotype, Mudd-Summers strain) were grown on BHK-21 cell monolayers and were purified by sucrose and tartrate gradients as previously described (24). Transcription-competent VSV cores were prepared as described before (16).

**In vitro polymerase assays and purification of RNA products.** Standard reaction mixtures (300 μl) contained the following: 50 mM Tris-acetate (pH 8.2); 8 mM Mg-acetate; 0.3 M K-acetate; 0.1% Nonidet P-40; 2 mM dithioerythritol or dithiothreitol; 16 U of RNasin (Promega); 30 μg of purified virions; ATP, GTP, and UTP (1 mM each); and 0.1 mM [$^3$H]CTP (ICN Pharmaceuticals; ~15 μCi per reaction). The effect of reaction pH was evaluated by changing the 50 mM Tris-acetate buffer component. The pH values indicated in all experiments refer to the pHs of the 50 mM buffers, not the final pHs of the reaction mixtures. Reaction mixtures were incubated for 2 h unless otherwise stated, and [$^3$H]CTP incorporation was determined as described before (24). The fractions of total products corresponding to N gene transcripts are ~40 and ~30% for wild-type and polR1 viruses, respectively (24). Each microgram of product synthesized under these conditions therefore contained an estimated eight copies of N mRNA for the wild-type virus and six copies for the polR1 mutant (30 μg of virions contains ~50 ng of N gene template).

RNA transcripts were purified by proteinase K digestion, phenol-chloroform extraction, and centrifugation through a Sephadex G-50 spin column as described previously (24). The latter step removes any transcript ≤20 nt long. To ensure complete recovery of all other transcripts, including leader, by ethanol precipitation, centrifugation was carried out in a Beckman TLA 100.3 rotor at 50,000 rpm for 30 min in the presence of 20 μg of glycogen carrier. Under such conditions, the 46-nt VSV defective interfering particle (DI) leader was fully recovered (33).

**Preparation of T7 riboprobe and 3′-end-labeled VSV genome RNA.** The T7 riboprobe was synthesized from plasmid pVSV3′(−) containing an insert corresponding to the first 109 bases from the 3′ end of the VSV genome. The insert sequence was amplified by PCR from the pTZ-JS77(+) plasmid (a gift from Tim Wong, University of Washington). The latter plasmid was constructed by inserting the *Pst*I fragment of plasmid pJS77 (32) into pTZ18R (Pharmacia). Primers for PCR amplification contained the first 17 nt of the VSV plus sense leader sequence and nucleotides 59 to 43 of the minus-sense N gene, preceded by a six-base *Eco*RI site and one 5′ terminal G residue. After being cut with *Eco*RI, the PCR product was cloned into the *Eco*RI site of pGEM1 (Promega) to yield pVSV3′(−).

pVSV3′(−) was linearized with *Bam*HI, which cuts 19 bases beyond the VSV insert. T7 transcripts from this template were 143 bases long and included 15 and 23 nt of vector sequence flanking the 5′ and 3′ ends, respectively, of the minus-sense VSV sequence (see Fig. 2A). The transcripts were synthesized in 50-μl reaction mixtures containing 15 mM $MgCl_2$; 40 mM Tris-Cl (pH 8.0); ATP, GTP, and CTP (1 mM each); 200 μM [α-$^{32}$P]UTP (NEN; ~200 μCi per reaction mixture); 5 mM dithioerythritol; 2 μg of linearized DNA template; 20 U of RNasin (Promega); and 50 U of T7 RNA polymerase (U.S. Biochemicals). After incubation at 37°C for 2 h, the DNA template and unincorporated label were removed by digestion with RNase-free DNase, phenol-chloroform extraction, centrifugation through a Sephadex G-50 spin column, and ethanol precipitation with 20 μg of glycogen carrier. The riboprobe was further purified on 7 M urea–8% polyacrylamide gels. End labeling of VSV genome RNA with cytidine 3′,5′-bis-[$^{32}$P]phosphate and T4 RNA ligase was carried out as described before (27), except for an additional purification of VSV genome RNA through a Sepharose CL-6B (Sigma) column prior to ligation.

**Ribonuclease protection assays.** Approximately 100 ng of purified VSV polymerase products was mixed with ~68 ng of the riboprobe in 9 μl of 10 mM Tris-Cl (pH 7.7)–1 mM EDTA. Extrapolating from our earlier findings (24), leader probe sequences in this mixture were present in a 30- to 60-fold molar excess over VSV leader and/or readthrough transcripts. The mixture was immersed in a boiling water bath for 1 min, after which 1 μl of 3 M NaCl was immediately added and the sample was incubated at 70°C for 30 min. It was then cooled slowly to room temperature by being placed on the bench top for 30 min. Digestion with 3.2 μg of RNase A (Sigma) and 1.2 U of RNase $T_1$ (Promega) was then carried out in a mixture with a total volume of 50 μl containing 0.1% SDS, 22.5 mM Tris-Cl (pH 7.7), 2.25 mM EDTA, and 1.22 M NaCl. After 2 h of incubation at room temperature (~22°C), the volume was adjusted to 200 μl with 10 mM Tris-Cl (pH 7.7)–1 mM EDTA buffer and Sarkosyl and proteinase K were added at 1% and 1 mg/ml final concentrations, respectively. After incubation at 55°C for 10 min and addition of 20 μg of glycogen carrier, the products were extracted with phenol-chloroform and precipitated overnight with ethanol. Centrifugation was carried out again as described above for 30 min at 50,000 rpm prior to electrophoresis on 7 M urea–8% polyacrylamide gels. The gels were fixed, dried, and exposed to film, and the bands were quantified by PhosphorImager analysis (Molecular Dynamics).

For the experiment testing the possible effects of virion-associated nucleases on transcript ratios, 100 ng of purified VSV products was incubated with virions under transcription conditions exactly as described above (except for omitting CTP and UTP) and was repurified as described above.

**Primer extension assays.** Chemically synthesized oligonucleotides (San Diego State University Microchemical Core Facility) mapping to positions 79 to 65, 154 to 140, 248 to 234, 444 to 430, and 950 to 935 of the N gene were labeled at their 5′ ends with polynucleotide kinase and [γ-$^{32}$P]ATP as described by Sambrook et al. (29) and were used one at a time for a parallel primer extension assay on wild-type and polR1 products purified as described above. Standard reaction mixtures (50-μl volume) contained 100 to 300 ng of VSV products, 15 ng of labeled oligonucleotides, 0.5 mM deoxynucleoside triphosphate, 80 mM NaCl, 8 mM $MgCl_2$, 50 mM Tris-Cl (pH 8.7 at 1 M), and 16 U of avian myeloblastosis virus reverse transcriptase (Promega). After incubation for 2 h at 42°C, the reaction mixture was adjusted to 0.3 M NaCl, 20 μg of glycogen was added, and the mixture was ethanol precipitated. Recovered products were analyzed on 7 M urea–4% polyacrylamide gels, and the bands were quantified by PhosphorImager analysis.

## RESULTS

**Assay of leader, N, and readthrough transcript synthesis by RNase protection.** Our earlier studies established that polR viruses synthesize a normal complement of mRNAs in vitro in addition to readthrough transcripts of the leader-N gene junction. These studies, as well as those of others, suggested that polR products also contain a smaller proportion of leader molecules than do wild-type products (12, 24, 25). A possible change in the ratio of leader to N gene transcription as a result of the polR mutation might bear important implications for the current 3′ end entry model of transcription in nonsegmented negative-strand viruses. We therefore set out to confirm these preliminary observations and to determine the precise molar ratios of transcripts synthesized by wild-type and polR1 mutant viruses in vitro by using an RNase protection assay (RPA) and an [α-$^{32}$P]UTP-labeled riboprobe containing the first 109 nt of the VSV genome template.

Figure 1 compares sequencing gel patterns of nuclease-resistant fragments obtained with wild-type and polR1 virus products after 0.5 h and 2 h of transcription in vitro. Four major groups of bands were generated, clustering at 45 and 46,

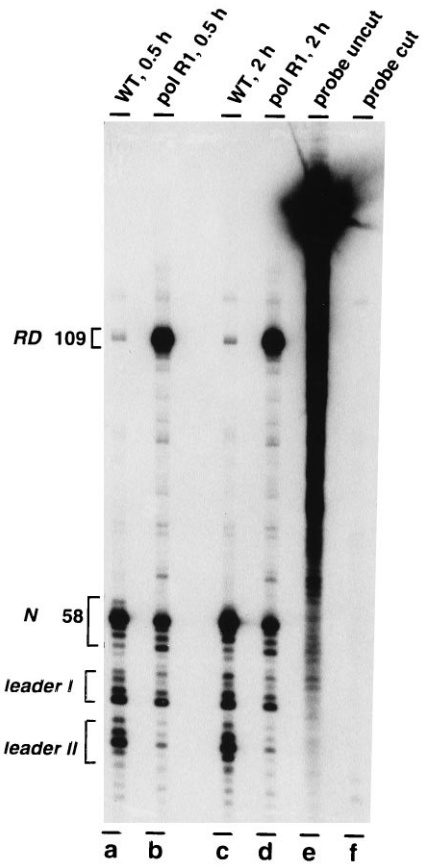



FIG. 1. Autoradiograph of RPA of wild-type (WT) and polR1 VSV products synthesized in vitro. $^{32}$P-labeled protected fragments were analyzed on sequencing gels (see Materials and Methods). Groups of fragments originating from leader RNA, N mRNA, and readthrough (RD) are indicated. Equal amounts of transcripts were analyzed in all lanes (for amounts synthesized, see text). Products were obtained after either 0.5 h (lanes a and b) or 2 h (lanes c and d) of in vitro synthesis. Also shown are the results obtained in the absence of virus products, either without (lane e) or with (lane f) RNases A and $T_1$.

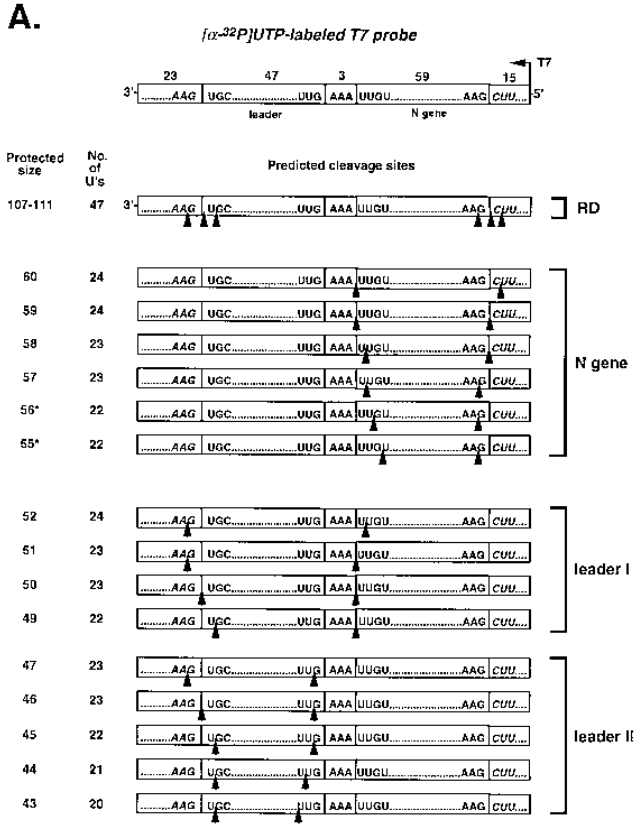
49 and 50, 58 and 59, and 109 nt (see below for precise size determinations). Two distinct groups of bands were anticipated for leader because of heterogeneous termination between positions 46 and 50 of the VSV genome (21). The 45- and 46-nt fragments were expected for leaders terminating at position 46, while fragments four nt larger (the three intergenic A residues are resistant to RNases A and $T_1$) were anticipated for leaders terminating anywhere between positions 47 and 50 (Fig. 2A shows the predicted cleavage sites). The spread of bands differing by one nucleotide in each fragment group was also expected and is a consequence of duplex "breathing" during nuclease digestion. A:U base pairs at the very ends of duplexes are particularly susceptible to nibbling by nuclease, as reflected in the pattern seen here.

In all RPAs reported here, we used an estimated minimum 30-fold molar excess of probe relative to leader RNA to ensure that the ratio of protected fragments accurately reflected the accumulation of transcripts. We verified that this was in fact the case by using higher probe-to-product ratios, which gave the same pattern (data not shown). Nuclease digestion of the riboprobe in the absence of VSV products yielded only trace amounts of resistant bands (Fig. 1, lane f), none of which interfered with the quantitation of VSV transcripts. The protection pattern was highly reproducible, the only significant variation being a change in the sizes of the major leader bands (46 versus 45 nt and 50 versus 49 nt) which likely reflected slight variations in the extent of nuclease digestion (see Fig. 3 below). We took special precautions to ensure that the small leader RNAs were quantitatively recovered from transcription reaction mixtures (see Materials and Methods). This was verified by performing RPAs on crude transcription reaction mixtures without product purification by using the guanidine isothiocyanate methodology of Haines and Gillespie (14). The latter approach produced similar results (data not shown), excluding the possibility that shorter leader RNAs of sufficient length to yield stable hybrids (12 to 15 nt) were excluded from our analysis. Our earlier analysis of CsCl-purified polR1 transcripts by high-resolution polyacrylamide gel electrophoresis analysis (25) or by RPA with a 3′-end-labeled genome probe (24) also showed no evidence of short leader transcripts.

Protected fragments obtained with polR1 virus transcripts differed from those obtained with wild-type transcripts in several important respects. The most obvious and expected change was the large increase in readthrough bands at ~109 nt (Fig. 1, lanes b and d). The second difference was a significant decrease in the amount of leader as a proportion of total products, confirming our earlier and more indirect evidence based on RPAs using a 3′-end-labeled genome probe (24). Unexpectedly, this decrease was far more pronounced for the shorter leader RNA terminating at base 46. The polR mutation therefore affects leader termination site preference at the junction, in addition to promoting antitermination. Readthrough bands smaller than 109 nt but larger than fragments derived from N mRNA were also present in small amounts in the polR1 products (Fig. 1, lanes b and d). This was also expected from our previous studies using the end-labeled probe, which showed heterogeneous termination of readthrough transcripts within the N gene. Most readthroughs, however, extend beyond 200 bases from the 3′ end (24; also see below), as reflected here by the far more intense bands at ~109 nt.

The relative amounts of leader, N, and readthrough in products from wild-type and mutant viruses remained exactly the same after 0.5 or 2 h of transcription in vitro (Fig. 1; compare lanes a and b to lanes c and d). This result is significant in regard to an earlier proposal that transcription complexes "frozen" at internal gene starts during virus packaging are capable of initiating transcription (9). Note that all of our RPAs were carried out with the same amount of products regardless of how much RNA accumulated in each reaction mixture (see Materials and Methods). No increase in band intensities was therefore seen after 2 h versus after 0.5 h of reaction. [$^3$H]CTP incorporation values, however, indicated that 0.55 and 0.63 μg of RNA accumulated after 0.5 h for wild-type and polR1 viruses, respectively. After 2 h, the corresponding values were 3.4 and 1.7 μg, respectively. From these amounts, we estimate that polR1 virus synthesized at least 10 copies of N mRNA in 2 h, in agreement with our previous studies (16, 25). Since multiple rounds of synthesis took place, a significant contribution from preloaded polymerase was very unlikely. Moreover, had there been such a contribution, a higher ratio of N to leader would have been expected at early versus late times of transcription.

**Identification of RNase-protected fragments originating from leader and readthrough by using a 3′-end-labeled genome probe.** The above results showed that leader transcript amounts relative to N mRNA were reduced in polR1 products. A cursory look at relative amounts of N and leader in Fig. 1 suggested roughly equimolar amounts for wild-type products but an excess of N over leader in the case of polR1 products



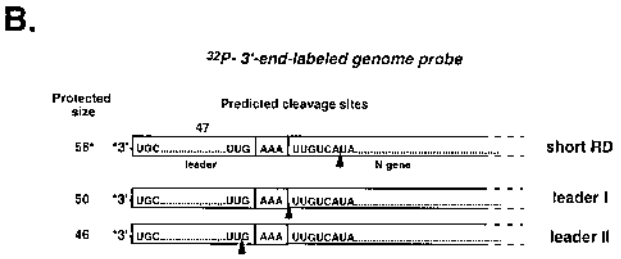




FIG. 2. Origins of protected fragments obtained by RPA with the [α-$^{32}$P]UTP-labeled riboprobe (A) and the 3′-end-labeled VSV genome probe (B). The 143-nt-long riboprobe, illustrated at the top of panel A, includes the first 109 nt at the 3′ end of the VSV genome flanked by 15 nt of vector sequence at the 5′ end and 23 nt at the 3′ end. Boxes delineate the 47-nt-long leader gene, the three intergenic A residues, the 59-nt-long N gene sequence, and flanking probe sequences. Predicted cleavage sites, due to the combined action of RNases A (pyrimide specific) and $T_1$ (G specific) on the riboprobe strand of duplexes originating from readthrough (RD), N gene transcripts, and leaders, are indicated by arrowheads. The sizes of the protected fragments and the proportions of uridine residues (for the riboprobe) are also indicated. The asterisk designation for the 55- and 56-nt fragments indicates a dual origin: N mRNA and short readthrough (see text).

(the number of uridine residues in both classes of protected fragments is roughly the same; see below). The latter, if true, put into question the validity of the 3′ end entry model which stipulates that leader must be synthesized prior to N mRNA. We therefore wanted to confirm the origin of each protected band before attempting to determine precise ratios of the transcripts by their radiolabel content. Accordingly, we compared the pattern of protected fragments using a 3′-end-labeled VSV genome probe alongside the uniformly labeled T7 riboprobe. All bands obtained with the end-labeled probe should thus clearly identify fragments generated by leaders. Figure 3 shows this comparison. The T7 riboprobe pattern (Fig. 3, lane a) was very similar to that seen in Fig. 1. The 55- to 60-nt group of bands, however, was essentially absent in the end-labeled probe pattern (Fig. 3, lane c). Leader bands were also shifted up 1 nt in the latter case. This was entirely expected because cleavage internal to base 1 due to duplex breathing should remove the end label (see Fig. 2B for predicted cleavages).

The results shown in Fig. 3 also provided the exact sizing of protected bands. The size marker lanes (b, e, and f) contained in vitro-labeled transcripts from a crude preparation of VSV copy-back DIs. The minus-strand leaders synthesized by these DIs are precisely 46 and 55 nt in length (31, 40) but migrate here as 47- and 56-nt bands because of their 5′-phosphorylated ends. The major leader-derived protected fragments in the RPA were therefore exactly 46 and 50 nt in length as determined by the end-labeled probe (Fig. 3, lane c) and 45 or 46 nt and 49 or 50 nt in length as determined by the T7 riboprobe (Fig. 3, lane a).

Protected fragments obtained with the end-labeled genome probe annealed to polR1 products confirmed the results of the previous T7 riboprobe analysis showing a lower proportion of leader II relative to leader I (Fig. 3, lane d). Short polR1 readthrough transcripts larger than leader I were also evident, as expected. One such readthrough band was 56 nt in length, indicating that the protected band of this size obtained with the T7 riboprobe likely originated partially from readthrough and partially from N mRNA (see below). Further confirmation of band assignments was also obtained by using two additional probes for RPA: a 3′-end-labeled riboprobe containing nt 11 to 109 of the VSV genome and a uniformly labeled riboprobe containing the first 214 nt of the genome. The end-labeled probe showed a change in leader bands only (10 nt shorter), while the uniformly labeled probe yielded an N-derived band of ~164 nt in place of the 58 or 59 nt band, as predicted (data not shown). The primer extension analysis described below also provided independent confirmation of the readthrough-to-N-mRNA-transcript ratios observed here by RPA.

Note that a nuclease-resistant fragment larger than readthrough was also seen here with wild-type products (Fig. 3, lane a). This nonspecific band was variably present in some riboprobe preparations in the absence of added VSV transcripts. Bands ~75 nt in length in the undigested end-labeled probe sample (Fig. 3, lane h) were likely due to end labeling of trace amounts of tRNAs packaged in virus particles (18). In any case, no nuclease-resistant bands were derived from this probe in the absence of added product (lane g).

**pH effect on transcript synthesis and further confirmation of RNase-protected fragment origins.** We previously documented small but significant differences in pH optimum for overall transcription of polR1 versus wild-type virus in vitro (26). Preliminary results obtained by using primer extension assays to measure N mRNA and readthrough suggested that higher pH favored readthrough. We therefore explored whether this parameter might prove useful in further distinguishing between leader, N, and readthrough transcripts. Figure 4 shows the effects of increasing the pH of the reaction buffer from 7.0 to 8.2 on the pattern of protected fragments obtained with the T7 riboprobe. Equal amounts of transcription products were used in the RPA as before. Overall transcription activity ([$^3$H]CTP incorporation) displayed a relatively broad optimum at pH 7.5 for both wild-type and polR1 virus and about threefold higher activity at pH 7.0 for the mutant (data not shown), in agreement with our previous findings (26). In-

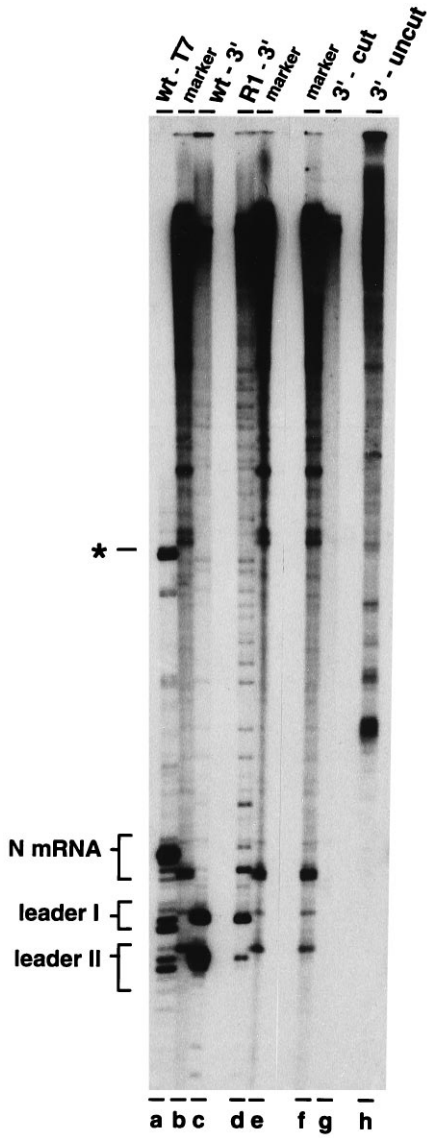



FIG. 3. Comparison of RPA carried out with an [α-$^{32}$P]UTP-labeled riboprobe (as described in the legend for Fig. 1) and RPA carried out with a 3′-end-labeled VSV genome probe. The latter probe identifies protected fragments originating solely from leaders and readthrough. Lane a, wild-type products and labeled riboprobe; lane c, wild-type products and end-labeled genome probe; lane d, polR1 products and end-labeled genome probe; lanes b, e, and f, [α-$^{32}$P]UTP-labeled products synthesized in vitro with a crude preparation of VSV DIs as size markers (the main bands in these lanes migrate as 47- and 56-nt bands [see text]); lanes g and h, 3′-end-labeled probe in the absence of VSV products with and without nuclease digestion, respectively. The asterisk indicates a nonspecific nuclease-resistant fragment occasionally obtained in the absence of VSV products with some riboprobe preparations.

creasing pH dramatically stimulated polR1 readthrough transcript synthesis relative to that of N mRNA (Fig. 4, lanes e to h). This was also true for the much smaller amounts of readthrough in wild-type products (Fig. 4, lanes a to d). PhosphorImager quantitation showed a 10-fold increase in readthrough relative to N mRNA (bands 57 to 60 nt) over the pH range examined. Both leaders taken together showed minimal changes in their ratio to N mRNA with increasing pH (25% increase for wild-type virus and 13% decrease for polR1 virus), while the leader-I-to-leader-II ratio increased 2.9-fold for wild-type virus and only 1.1-fold for polR1 virus. Taken together with the fact that total 3′-end initiations in polR1 (leader plus readthrough) increased only 38% between pH 7.0 and 8.5 (adjusted for total activity per template), these results indicate that the increase in readthrough was, for the most part, at the expense of leader and N mRNA. When compared to total 3′-end initiations rather than to N mRNA, polR1 readthrough increased 3.7-fold (20 to 73%). We also examined products synthesized at pH 8.5 and noted a further slight increase in readthrough/N and leader I/leader II ratios (data not shown).

Note that the overall pattern of protected fragments in Fig. 4 was very similar to that in Fig. 1, except for a slight increase in the proportion of the 46- and 50-nt bands to the 45- and 49-nt bands, respectively. More importantly, the pH results provided helpful information on the origins of the minor 55-nt and 56-nt bands. For wild-type products, these bands clearly originated from N mRNA, but as noted above in connection with the end-labeled probe, polR1 products also contained a short 56-nt-long readthrough fragment (Fig. 3, lane d). The 56-nt band from polR1 products analyzed with the riboprobe should have therefore originated from two sources: N mRNA and the short readthrough. The latter should have also given rise to a 55-nt band because of duplex breathing, and this should only be detectable when using the riboprobe (Fig. 2B). The dual origin of bands 56 and 55 was in fact evident in Fig. 1 and also in Fig. 4, since polR1 products generated larger amounts of these bands relative to the major N bands (57 to 60 nt) than did the wild-type products. As expected from a dual origin, the effect of pH on bands 56 and 55 was less dramatic than on the long readthrough at ~109 nt (2-fold rather than

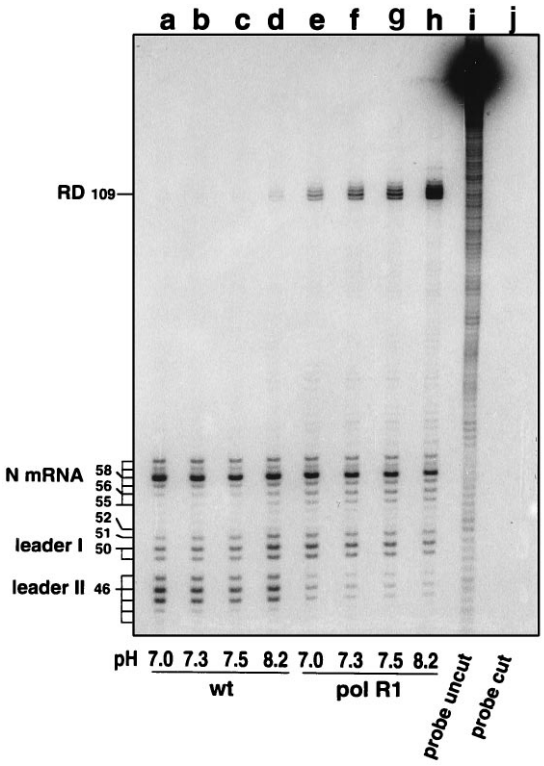



FIG. 4. Effect of pH on wild-type and polR1 products analyzed by RPA. Analysis was carried out as described in the legend for Fig. 1 (2-h reactions) except that the pH of the reaction mixtures was varied. The indicated pH values refer to the Tris buffer component and thus only approximate those of the total reaction mixtures. Protected fragment sizes and origins are indicated on the left. wt, wild type.

TABLE 1. Molar ratios of leader, N gene, and readthrough transcripts synthesized by wild-type and polR1 VSV in vitro[a]

| Expt no. | N/leader | | Readthrough/N | |
|---|---|---|---|---|
| | wt[b] | polR1 | wt | polR1 |
| 1 | 1.0 | 2.0 | 0.03 | 1.4 |
| 2[c] | 0.9 | 1.8 | 0.05 | 2.1 |
| 3 | 1.3 | 2.0 | 0.05 | 1.6 |
| 4 | 1.1 | 1.7 | 0.05 | 1.6 |
| 5 | 0.8 | 2.0 | 0.06 | 1.7 |
| 6 | 1.1 | 2.0 | 0.07 | 1.8 |
| 7 | 1.1 | 1.9 | 0.07 | 1.4 |
| Mean ± SD | 1.0 ± 0.1 | 1.9 ± 0.1 | 0.05 ± 0.01 | 1.7 ± 0.2 |

[a] Molar ratios were determined by RPAs as described in the legend for Fig. 1 by using PhosphorImager values for protected bands corresponding to leader, N, and readthrough transcripts, corrected for the number of uridine residues in each fragment (see text for details).

[b] wt, wild type.

[c] Experiment 2 was carried out with purified transcription-competent virus cores instead of whole virus.

10-fold). Furthermore, RPA of polR1 transcripts using $T_1$ RNase only (cleavage after G residues exclusively) yielded a major protected fragment of 56 nt (data not shown) which could have originated only from N mRNA (Fig. 2A). For determinations of N/leader molar ratios in polR1 products, we therefore subtracted the contributions of the short readthroughs from the totals in bands 55 to 60. This could easily be done since wild-type products gave us the proportion due to N mRNA only. In Fig. 4, 88.6% of the total radioactivity in bands 55 to 60, at the pH routinely used in our assays (pH 8.2), was derived from N mRNA. Independent determinations of this value for two additional polR1 transcript preparations yielded values of 85.1 and 93.8%.

**Molar ratios of leader, N, and readthrough transcripts from wild-type and polR1 viruses.** The above results reassured us of correct identification of all protected fragments generated with the T7 riboprobe. We therefore assigned bands 107 to 111 to readthrough, 43 to 47 and 49 to 52 to leader, and 55 to 60 to N mRNA. After subtraction of background obtained from the lane containing digested probe only (negligible in almost all cases), PhosphorImager values for each individual band (except for readthrough, which we determined as a group) were converted to relative molar amounts on the basis of the band's content of labeled uridine residues. Inferred cleavage sites and the numbers of uridine residues for all bands used in this quantitation are summarized in Fig. 2A. For polR1 products, the values in bands 55 to 60 were corrected for the readthrough component as described above. The results obtained by using seven different transcription product preparations and two independent sources of purified wild-type and polR1 viruses are shown in Table 1. Ratios for experiments 1 to 3 were determined by summing the contributions of individual bands as detailed above, while for experiments 4 to 7, bands 43 to 52 nt (leader) and 55 to 60 nt (N mRNA) were grouped for PhosphorImager quantitation. Adding band 48 to the leader group had no significant consequence since it was never present in more than trace amounts, if at all (Fig. 1, 3, and 4). For polR1 bands of 55 to 60 nt, the N mRNA contribution in experiments 4 to 7 was taken as the average of the three determinations in experiments 1 to 3 (89%).

Table 1 indicates that N/leader molar ratios varied from 0.8 to 1.3 for wild-type virus, with a mean ± standard deviation (SD) of 1.0 ± 0.1. If the polymerase can only start productive transcription at the 3′ end of the template, these results indicate that reinitiation at the N gene must take place with close to 100% efficiency. In contrast, N/leader ratios for polR1 virus ranged from 1.7 to 2.0, with a mean ± SD of 1.9 ± 0.1. This strongly suggests that synthesis of leader before N mRNA is not required, at least in the case of polR1. Readthrough/N ratios varied from 0.03 to 0.07 for wild-type virus and from 1.4 to 2.1 for polR1 virus, with means ± SDs of 0.05 ± 0.01 and 1.7 ± 0.2, respectively. The somewhat greater variation of readthrough/N ratios compared to N/leader ratios could well be due to small variations in reaction mixture pH, which have a much larger effect on readthrough than leader (Fig. 4).

We have also confirmed by RPA our earlier finding that low ATP concentration, 50 μM in lieu of 1 mM, decreases N mRNA synthesis relative to leader synthesis for wild-type virus but much less so for polR1 virus (2). Raising ATP concentration to 3 mM, however, had little or no effect on transcript ratios (data not shown).

**Processivity of wild-type and polR1 virus polymerases copying the N gene is similar.** We showed previously that polR mRNA transcripts synthesized in vitro are identical in size and relative amount to those produced by wild-type virus, except for slightly longer poly(A) tails (25). However, the RPAs described above only probed the first 59 nt of the N gene. If a substantial fraction of polR1 N gene transcripts terminated prematurely beyond position 59, as readthrough does, these could conceivably have escaped detection in our earlier study because of size heterogeneity. To test this possibility, we carried out a series of primer extension analyses using five different end-labeled oligonucleotides whose 5′ ends mapped to positions 79, 154, 248, 444, and 950 along the N gene. We anticipated that gel analysis of products extended by reverse transcriptase would show band sizes equal to the distance between the primer and the N gene start for N mRNA and bands 50 nt longer for readthrough. Figure 5 illustrates this expected outcome for primers mapping to positions 248 and 950 of the N gene. N mRNA and readthrough bands were well separated from each other, and their amounts increased in proportion to the amounts of VSV transcripts. This assay could thus be used to obtain relative amounts of N and readthrough transcripts of a given length in wild-type versus polR1 products.

Figure 6 shows a plot of the ratio of polR1 N mRNA to wild-type N mRNA (PhosphorImager values) as a function of primer position derived from several independent experiments. As a fraction of total products, polR1 N transcripts were 50 to 60% as abundant as wild-type N transcripts, in agreement with RPA results (Fig. 1 and 4). More importantly, the ratio of polR1 to wild-type N mRNA remained essentially constant at all primer positions. The length distribution of N gene transcripts, which is a measure of the processivity of the polymerase responsible for this synthesis, was therefore essentially identical for both wild-type and polR1 viruses. We conclude that the protected bands generated from N mRNA in the above RPA experiments reflect synthesis of full-size N transcripts and not of prematurely terminated products. Additional support for this conclusion was also obtained by carrying out RPA with a riboprobe containing the first 214 nt of the genome; this yielded N/leader transcript ratios similar to those reported above (33).

In contrast to N mRNA synthesis, the processivity for readthrough transcript synthesis in polR1 was clearly diminished, since the ratio of readthrough to wild-type N mRNA approximated 1.0 until position 248 and then decreased to about 0.6 at position 444 (Fig. 6). The behavior of readthrough in this assay was thus very similar to that revealed in our previous results obtained with the end-labeled genome probe and showing an abundance of nuclease-protected fragments ~200 to

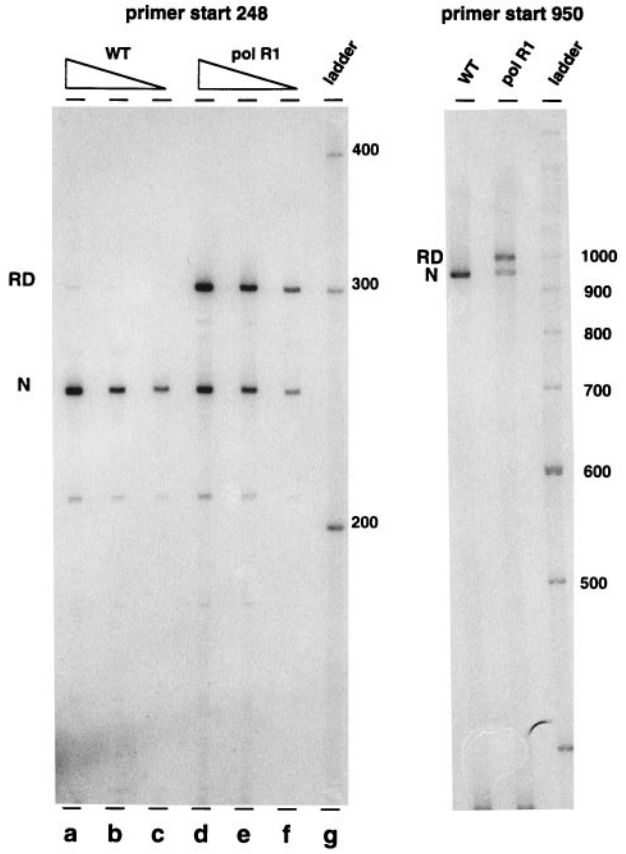



FIG. 5. Primer extension analysis of transcripts complementary to the N gene sequence synthesized in vitro by wild-type (WT) and polR1 virus. The left panel shows an autoradiograph of extension products obtained with reverse transcriptase and a 5′-end-labeled primer mapping to positions 248 to 234 of the N gene and analyzed on a 4% sequencing gel (see Materials and Methods). The amount of products was lowered in threefold increments (~300 to ~30 ng) in lanes a to c and d to f. The right panel shows the same analysis with ~100 ng of products and a primer mapping to positions 950 to 935 of the N gene. The positions of extension products corresponding to N mRNA and readthrough (RD; 50 bases longer) are indicated. Size marker lanes (ladder) contained end-labeled 100-bp DNA ladders.

400 nt in size (24). The polymerase that starts at the leader on the polR1 template is thus less processive.

Note that the primer extension assays described here would not detect abortive N gene transcripts smaller than ~75 nt. The RPAs, on the other hand, covered the range from ~20 nt (cutoff limit of Sephadex G-50 spin-column purification) to 164 nt. The previously described abortive N gene transcripts 11 to 14 nt long (28) are therefore irrelevant to our estimates of productive syntheses of leader and N transcripts and the molar ratios reported in Table 1. However, we cannot rule out the possibility that a small fraction of the protected fragments scored as leader by RPA in fact originated from abortive N transcripts 43 to 49 nt long. This is, however, unlikely since the pattern of leader fragments obtained with the end-labeled genome probe was very similar to that obtained with the uniformly labeled riboprobe (Fig. 3), and transcripts of this size were not present in either wild-type or polR1 products analyzed previously on gels (25). In any case, if a small fraction of leader-sized fragments did in fact originate from abortive N transcripts, the values reported in Table 1 may slightly underestimate authentic N/leader molar ratios.

**Leader deficiency in polR1 virus products is not due to preferential degradation.** The fact that transcription products obtained after 0.5 h and those obtained after 2 h of reaction in vitro displayed the same ratio of transcripts (Fig. 1) strongly argued against the possibility that leader is preferentially degraded by virion-associated nucleases. Moreover, identical results were obtained when using transcription-competent virus cores purified of their envelope components (Table 1). Nonetheless, we addressed this concern directly by determining N/leader ratios in purified transcription products before and after incubation with virions. The purified transcripts were incubated under the same conditions as those used for their synthesis except for the absence of CTP and UTP.

The results of this nuclease test are shown in Fig. 7. The control, showing the pattern of protected fragments from purified polR1 products before incubation with polR1 virions, is shown in lane d. The N/leader molar ratio of this preparation was 2.1. The effect of incubating this same preparation at 30°C for 2 h under transcription conditions without virions (lane c), with virions at 0°C (lane b), or with virions at 30°C (lane a) was minimal. After all three conditions of incubation, the N/leader ratio was 1.8. The small loss of N transcripts observed was likely due to trace nuclease activity in transcription reaction components since it occurred in the absence of virions (lane c). No detectable change in N/leader ratio due to transcription reaction components was observed in other experiments (data not shown). Likewise, no significant loss of leader was observed for wild-type products incubated with wild-type virions. The N/leader ratio in this case was 1.3 before incubation (data not shown) and 1.2 following incubation (Fig. 7, lanes d and e). We therefore conclude that leader RNA is not preferentially degraded by virion-associated nuclease(s) for either wild-type or polR1 virus. The transcript ratios determined by RPA must therefore closely reflect N mRNA and leader synthesis.

## DISCUSSION

The experiments reported here were carried out primarily to examine the effects of the polR mutation on leader and N gene transcription. We suspected that other aspects of the transcription process aside from readthrough of the leader-N gene

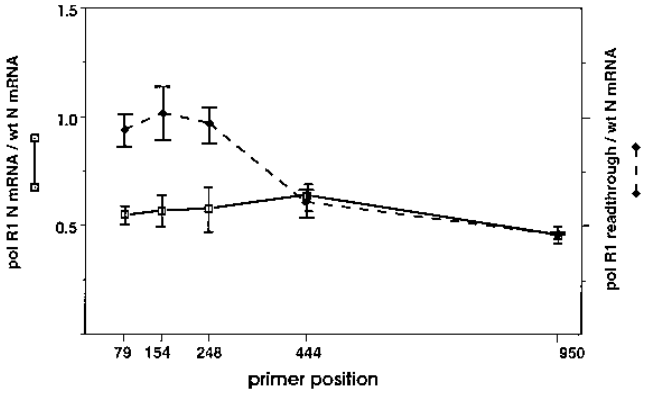



FIG. 6. Relative processivity of N mRNA and readthrough transcript synthesis by wild-type (wt) and polR1 virus in vitro. Products were analyzed by primer extension as described in the legend for Fig. 5, and the ratio of label (PhosphorImager values) in the N band derived from polR1 products to that derived in parallel from wild-type products is plotted as a function of primer position within the N gene. Each data point represents the average and standard error of two to five different experiments, depending on the primer. The ratio of polR1 readthrough to wild-type N mRNA from the same set of experiments is also shown.

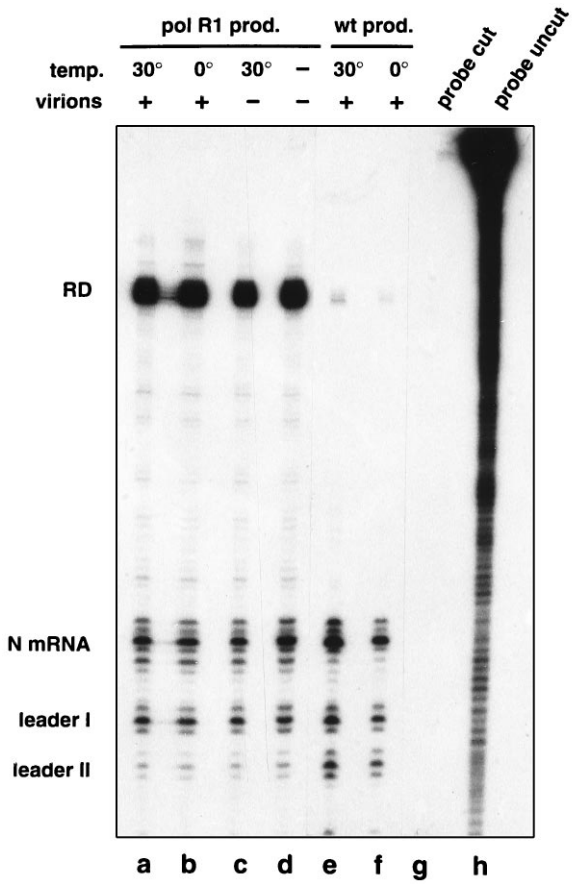



FIG. 7. Effect of incubation of purified wild-type (wt) and polR1 products with virions. Purified products were incubated at either 30 or 0°C or were not incubated (lane d) with the same virus preparation employed for their synthesis and under the same transcription conditions, except for the absence of CTP and UTP. Following incubation, products were repurified and analyzed by RPA as described before. Lanes g and h show RPA results in the absence of products with and without nuclease, respectively.

junction were affected by this mutation. What we found leads us to question some key aspects of the current model of transcription for VSV and, by implication, for other nonsegmented negative-strand RNA viruses. The polR mutation caused a nearly twofold increase in N mRNA synthesis relative to leader synthesis in vitro. This result confirms and extends earlier and more indirect evidence for this effect (12, 24, 25). More surprising, however, were the numbers obtained for N/leader molar ratios, i.e., 1.0 ± 0.1 for wild-type virus and 1.9 ± 0.1 for polR1 virus. These ratios not only indicate a lack of attenuation at the leader-N gene junction but also cast serious doubts on the requirement for the polymerase to synthesize leader before mRNAs. Remarkably, very similar N/leader molar ratios, also determined by RPAs, were reported for transcripts from the Sendai virus Z and H strains synthesized in vitro (37). The H strain, which produces little readthrough synthesis, like wild-type VSV, generated slightly more leader than N (120 ± 13%), while the Z strain, which reads through like VSV polR, synthesized about half as much leader as N (62 ± 4%). Since much smaller amounts of leader were detected in vivo, presumably due to high turnover, and since host nucleases could well be associated with virions, the authors cautiously concluded that roughly equimolar amounts of leader and N transcripts were synthesized by the polR-like Z strain.

Before concluding that N transcripts were in fact synthesized to a greater extent than leader by polR virus, it was incumbent upon us to rule out spurious explanations for the ratios. We took as many precautions as possible to ensure the validity of our findings. First, all transcripts copied from the 3′ end of the genome, i.e., leader, N, and readthrough, were measured simultaneously by RPA. We made certain that all transcription products, including the small leader molecules, were quantitatively recovered from transcription reactions. The origin of each protected band derived from the riboprobe containing the first 109 nt of the VSV genome was not only deduced on the basis of predicted sizes (Fig. 2) but was also confirmed by using an end-labeled genome probe (Fig. 3) and by probing the effects of pH on the transcription reaction (Fig. 4). Other supporting data not shown here included RPAs using RNase $T_1$ only and RPAs employing a uniformly labeled riboprobe containing the first 214 nt of the genome or a 3′-end-labeled riboprobe containing nucleotides 11 to 109 of the VSV genome. The determinations were repeated numerous times with independent preparations of each virus over the course of more than a year with fully consistent results.

We next considered whether N transcript amounts assayed by RPA reflected full-size N mRNA or abortive products terminating beyond the first 164 nt. Iverson and Rose (19) long ago reported that the processivity of the VSV polymerase within gene boundaries is very high since essentially equimolar synthesis of 5′ versus 3′ proximal regions of N, P, M, and G transcripts was observed in vitro. The question here was whether the polR virus mutation imparted a reduced processivity to the P-L complex synthesizing N gene transcripts. Primer extension assays using a nested set of oligonucleotides mapping to different positions along the N gene (59 to 950) clearly showed that the ratio of polR1 to wild-type N transcripts remained the same for all oligonucleotides (Fig. 6), ruling out abortive termination. Moreover, no abortive N gene transcripts were evident in wild-type or polR1 products analyzed by agarose gel analysis, either for this study (data not shown) or previously (25). The possibility that a substantial fraction of readthrough-synthesizing polymerases could "back up" to initiate internally at the N gene also appears very unlikely since higher readthrough frequency did not correlate with higher N-to-leader ratios (Table 1; Fig. 4).

We then gave consideration to the possibility that leader was preferentially degraded by a virion-associated nuclease during transcription. Two observations argued against this prospect: N/leader ratios were not affected by varying the duration of transcription reactions (Fig. 1), and purified virion cores lacking envelope components generated the same ratios (Table 1). Nonetheless, we tested this directly by incubating purified transcripts with disrupted virions under transcription conditions (Fig. 7). We found only a minor loss of N mRNA and no loss of leader upon incubation, suggesting that our RPAs may slightly underestimate N/leader transcript molar ratios.

Lastly, we entertained the possibility that pre-positioned polymerase complexes were responsible for the high N/leader ratios. This was clearly not the case, however, because such complexes would have been displaced by the several rounds of synthesis occurring under our transcription conditions. Moreover, if such complexes had, in fact, contributed to excess N, the N/leader ratio should have decreased with increasing time of synthesis (Fig. 1).

If the measured ratios do indeed reflect transcript synthesis, as our data forces us to conclude, what does this tell us about transcription and replication? First, it should be emphasized that the polR virus phenotype uncovered here does not imply that the leader sequence is dispensable for initiation of the

transcription process, only that prior synthesis of a leader transcript for every transcription start at the N gene is unnecessary. Second, our findings also do not imply that productive synthesis of mRNAs other than N can initiate internally. We have no reason to doubt that sequential synthesis is the rule once the polymerase starts at the N gene. Third, it is of course conceivable that only VSV polR shows productive internal initiation at the N gene, while wild-type virus follows the leader-first rule.

The crucial evidence implicating leader-before-N transcript synthesis for wild-type VSV transcription rests on the findings of Emerson (9), who used an in vitro-reconstituted system and partial-reaction conditions (see the introduction). Although these studies, carried out under partial-reaction conditions (absence of UTP and GTP), do provide evidence for the leader-first model, reiterative abortive initiations may not reflect productive synthesis, as suggested in a later conflicting report for which a similar reconstitution assay was used (36). In the case of our present polR VSV studies, we cannot rule out the possibility that synthesis of very short leader transcripts ($\leq$12 nt) is required prior to initiation at the N gene. However, if this were the case, synthesis of at least half of all polR1 N mRNA would require the polymerase to translocate to a site at least 38 nt upstream without dissociating from the template and without copying the rest of the leader gene sequence. Apparently, the VSV polymerase could not do this on a wild-type template under the partial-reaction conditions used by Emerson (9).

Although equimolar synthesis of leader and N gene transcripts, as seen here with wild-type virus, is compatible with the leader-first model, there are good reasons for suggesting that internal initiation could well take place in this case also. What causes excess N mRNA synthesis in polR1 is a template-associated N protein mutation, not a change in the RNA sequence anywhere near a polymerase entry site (6, 12, 24). The creation of a new entry site simply as a result of this protein mutation is highly unlikely. We therefore propose that wild-type VSV also possesses the same two polymerase entry sites. The polR mutation can then be viewed as causing a shift in the balance towards internal initiation. This two-site model, which by implication should also apply to other nonsegmented negative-strand RNA virus polymerases, is illustrated in Fig. 8. A single P-L polymerase complex could be responsible for both leader and N gene entry, or alternatively two different complexes might be involved. The latter possibility has been suggested before (3, 4, 5, 7, 34) and is consistent with the results of several studies showing uncoupling of the two types of synthesis in vitro (see the introduction). Polymerase complexes could conceivably differ in subunit composition and/or P phosphorylation, but this clearly remains speculative.

Assuming that leader synthesis also represents the first step in replication, it is still unclear how suppression of leader termination takes place, either in response to the P-N assembly complex in vivo or as a result of the polR mutation. The earlier model postulating that the distinction between a replicase and a transcriptase manifests itself only when a polymerase crosses the leader-N gene junction (20, 37) is clearly incompatible with our findings reported here. Internal initiation of transcription implies that this distinction is made before synthesis begins.

Curiously, we also found here that readthrough synthesis relative to N or leader synthesis was strongly stimulated by a modest increase in reaction pH (10-fold increase between pH 7.0 and 8.2). This was true not only for polR1 virus but also for the very low level of readthrough synthesis in wild-type virus. Since the polR phenotype is caused by a single charged amino substitution (Arg179 to His) in the template-associated N protein (7, 15, 24), the effect of pH suggests that a charge interaction between the template-associated N protein and the P-L polymerase complex may be crucial in determining antitermination at the leader-N gene junction and in switching to replication.

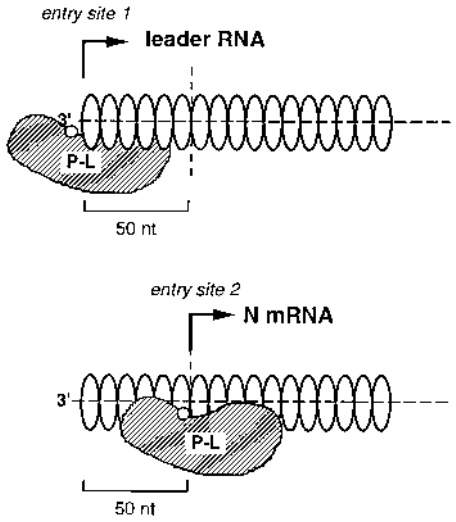



FIG. 8. Two polymerase entry sites model. Sequential transcription of mRNAs is proposed to initiate internally at the N gene start sequence, while leader RNA synthesis is initiated independently at the 3′ end of the N protein-encapsidated RNA template. The start site of synthesis, 50 nt apart in the two cases, is presumed to be positioned near the catalytic center of the P-L polymerase complex (illustrated here as a small open circle) when synthesis begins. The same P-L complex could conceivably be responsible for both types of synthesis or, alternatively, two different forms of P-L complexes might be involved (see text).

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