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In Vitro Transcription of Vesicular Stomatitis Virus: Initiation with GTP at a Specific Site Within the N Cistron

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In vitro transcripts of vesicular stomatitis virus (VSV) were either 5'-terminally labeled by incorporation of [β-^{32}P]GTP or were selected on Hg-agarose after incorporation of γ-thio-GTP. Capped RNAs ranged in size from 23 nucleotides, the shortest capped RNA detected, to full-length message size. The 5'-terminal sequences corresponded to those of N message and to a small amount of NS message. Approximately 14% of the capped *N* gene transcripts were terminated at positions 86 to 90 of the VSV genome, giving rise to specific, 36 to 40-nucleotide-long, capped RNA species. The GTP-initiated RNAs were short with a predominant 28-nucleotide-long RNA species. A minor portion was as large as mRNAs. Nucleotide sequence analyses of the short RNA revealed that it was specifically initiated at positon 91 of the VSV genome, 41 nucleotides within the N cistron. This corresponds exactly to the site where transcription of the 40-nucleotide-long, capped RNA terminated. Initiation with GTP at position 91 occurred at approximately the same frequency as termination of the capped RNA at position 90, suggesting that intracistronic initiation at position 91 may depend upon termination of transcription of the 5'-proximal region and therefore may be sequential. This unique RNA represents the first transcript of VSV which was initiated at an intracistronic site with GTP, and may also represent the first example of a transcript derived from a stop/start mechanism of VSV transcription in vitro. Although initiation occurred frequently at the beginning of the N cistron yielding 11 to 14-nucleotide-long, [β-^{32}P]ATP-labeled transcripts (D. F. Pinney and S. U. Emerson, J. Virol. **42**:889–896, 1982), capping of these short RNAs was not detected. This suggests that transcripts may have to be 15 to 23 nucleotides long to be accepted as substrates by the guanyltransferase.

The negative-sense RNA genome of vesicular stomatitis virus (VSV) codes for five structural proteins in the order of N-NS-M-G-L. Transcription is sequential (1, 3), and five discrete polyadenylated mRNAs have been detected in vivo as well as in vitro (for review see 4). Heterogeneous termini have been described for each individual message. About 85% of the transcripts begin with ^{7m}Gppp(m)AmpApCp . . . and ^{7m}Gppp(m)AmpmAmpCp. . . . The remaining 15% begin with either pppAp . . . or pppGp . . . in approximately equal amounts (21). In addition to the capped and pppAp . . . terminating RNAs, in vitro transcripts have been reported which were initiated with either pppGpAp . . . , pppGpCp . . . , or pppGpGp . . . . (12). Whereas the capped and pppAp . . . termini were derived mainly from the messages and leader RNA (8, 12, 20), the origins of transcripts initiated with GTP are unknown. In this communication, we report the first VSV in vitro transcription product that was initiated with GTP, and we identify its specific intracistronic initiation site on the VSV genome.

MATERIALS AND METHODS

Isolation and radioactive labeling of VSV transcripts. Throughout this study the Mudd-Summers isolate of VSV was used. In vitro transcription was carried out using detergent-disrupted virus (7). Transcripts were labeled with either [β-^{32}P]GTP or [β-^{32}P]ATP, which were prepared with 10 mCi of $^{32}P_i$ by the procedure by Kaufmann et al. (14). γ-Thio-GTP–initiated RNAs were selected either on Hg-agarose, provided by Ru Chih C. Huang (29), or on Affi-gel 501 (Bio-Rad, Richmond, Calif.) (5, 16a). These transcripts were internally labeled with [α-^{32}P]CTP or were labeled at their 3' termini with ^{32}P-cytidine 3',5'-bisphosphate and RNA ligase (10). [^{3}H]uridine-labeled in vivo transcripts were isolated from VSV-infected BHK-21 cells as previously described (27), except that nuclei-free cell extracts were applied directly to 10 to 30% sucrose gradients containing 0.5% sodium dodecyl sulfate.

Nucleotide sequence analyses. Transcripts were sepa-

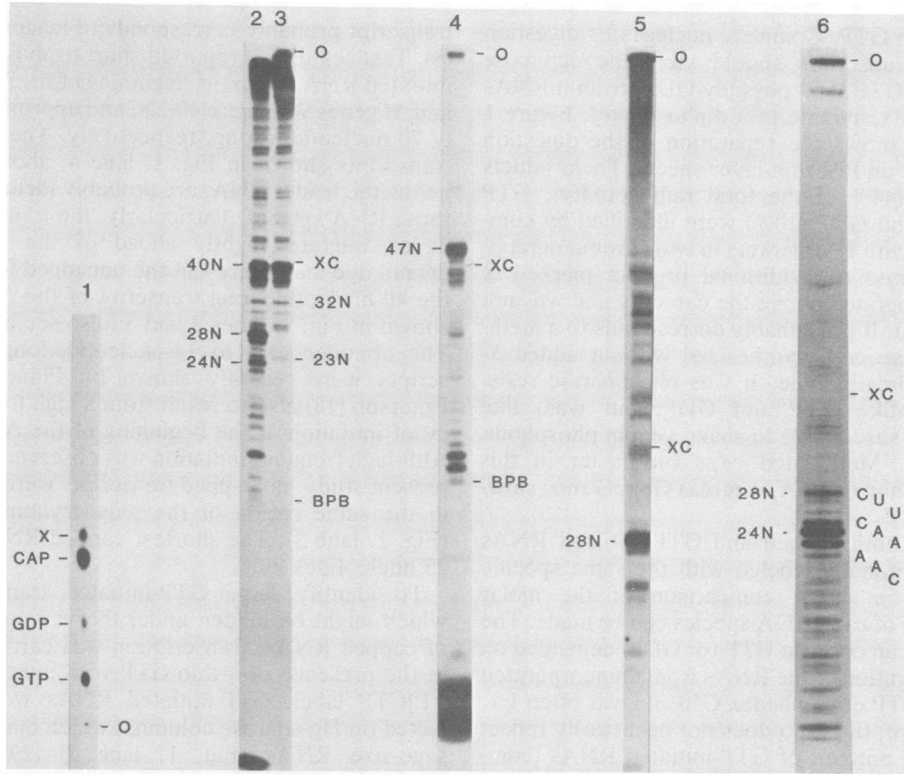

FIG. 1. Isolation of capped and GTP-initiated VSV in vitro transcripts. Lane 1, Complete nuclease P1 digest of [β-^{32}P]GTP-labeled RNAs (as shown in lane 2) separated on a PEI thin-layer sheet. Lanes 2 and 3, [β-^{32}P]GTP-labeled transcripts separated on a 12% polyacrylamide gel before (2) and after (3) phosphatase treatment. Lane 4, [β-^{32}P]ATP-labeled transcripts. Lane 5, [α-^{32}P]CTP-labeled transcripts synthesized in the presence of γ-thio-GTP and selected on Hg-agarose. Lane 6, γ-Thio-GTP-containing transcripts selected on Affi-Gel 501 and labeled at their 3′ termini with ^{32}P-cytidine 3′,5′-bisphosphate and RNA ligase. The results of nearest-neighbor analyses of the individual RNA species were as shown beside the RNA bands. The length of the transcripts (N = nucleotides) was determined by partial alkaline hydrolysis and separation of the fragments on polyacrylamide gels. The positions of the dyes xylene cyanol (XC) and bromophenol blue (BPB) were as indicated.

rated on 12% polyacrylamide gels (9). [β-^{32}P]GTP-labeled RNAs were either applied directly or after treatment with calf intestinal phosphatase. RNAs were eluted by crushing and soaking the gel slices. Partial hydrolysates were obtained by incubation for 1 h at 100°C in formamide (28) or by partial digestion with RNase T_2. Fragments were separated on one- or two-dimensional gels (26). Complete nuclease P1 and RNase T_2 digestions were carried out as previously described (13, 25). The products were separated by ionophoresis on PEI thin-layer sheets (16).

Hybridization of in vivo transcripts to cloned VSV DNA. A double-stranded DNA copy of the precise 3′ terminus of the VSV genome up to approximately position 370 was prepared and cloned into the *Escherichia coli* plasmid pBR322. The preparation and structure of this plasmid, pJS77, will be described elsewhere. The VSV insert in pJS77 was excised with *Pst*I and cleaved with *Bgl*II at a position corresponding to position 214 on the VSV genome (11, 15). The fragments were phosphatase-treated, labeled with [γ-^{32}P]ATP and polynucleotide kinase, and separated on a 7.5% polyacrylamide gel. The 5′-terminally labeled 214-nucleotide-long fragment {plus oligodeoxyguanidylic acid [oligo(dG)]·oligodeoxycytidylic acid [oligo(dC)] tail} was eluted from the gel slice and denatured, and the minus-sense, single-stranded DNA containing the oligo(dC) tail was specifically selected on oligo(dG)-cellulose. In vivo transcripts were annealed to an excess of the single-stranded DNA probe in 0.4 M NaCl–10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8) at 45°C for 16 h. Single-stranded RNA and DNA were digested with 1.25 U of nuclease S1 per μg of tRNA at 45°C for 30 min. The resistant duplex was separated on a 7.5% polyacrylamide gel next to an *Hae*III digest of φX174 DNA.

RESULTS

Identification of capped GTP- and ATP-initiated RNAs. In vitro transcripts of VSV were terminally labeled by incorporation of [β-^{32}P]GTP. Unlike most other viral and eucaryotic mRNAs, VSV messages conserve the beta-phosphate of the GTP in the cap (2). Therefore, labeling of the transcripts with [β-^{32}P]GTP specifically identifies capped RNAs and RNAs initi-

ated with GTP. Complete nuclease P1 digestion of the transcripts should yield the cap core GpppA, GTP, and possibly GDP (from mRNAs that may terminate in a diphosphate). Figure 1 (lane 1) shows the separation of the digestion products on PEI thin-layer sheets. The products GpppA (44% of the total radioactivity), GTP (31%), and GDP (10%) were identified by comparison with UV markers in two chromatographic systems. An additional product marked X (15%) migrated above the cap core and was not identified. It presumably corresponds to a methylated cap core synthesized without added *S*-adenosylmethionine. It was phosphatase resistant, unlike GTP and GDP, but was, like GpppA, susceptible to snake venom phosphodiesterase. Methylated caps run faster in this system than GpppA, whereas GpppG runs slower.

Since both capped and GTP-initiated RNAs were terminally labeled with the same specific activity, a direct comparison of the molar amounts of each RNA species can be made. The ratio of cap cores to GTP (or GDP) depended on the separation of the RNAs from unincorporated [β-^{32}P]GTP on Sephadex G50 and was often 1:1. Therefore, the ratio does not necessarily reflect the total amount of GTP-initiated RNAs (compare Fig. 1, lanes 2 and 3).

Equal portions of the transcripts were applied on 12% polyacrylamide gels either without phosphatase treatment or after phosphatase treatment to remove terminal phosphates (Fig. 1, lanes 2 and 3). Most of the label in the short transcripts was removed by the phosphatase treatment, including that on the predominant 28- and 24-nucleotide-long RNA species, indicating that they were initiated with GTP. On the other hand, the label in most of the larger transcripts (bigger than 23 nucleotides and up to message size) remained intact, demonstrating that they mostly represent capped RNA species. The shortest capped RNA species detected was 23 nucleotides long. Its migration did not change upon phosphatase treatment, suggesting that it did not contain a 3′- or 2′-terminal phosphate as a result of degradation of larger products. We would like to point out that capped RNA species run slower than triphosphate-ended RNA species of the same length. Elution of the major RNA species from gel slices and subsequent nuclease P1 digestions confirmed the presence of either terminal cap or GTP (data not shown).

Labeling of the transcripts with [β-^{32}P]ATP, on the other hand (Fig. 1, lane 4), yielded only short, up to 47-nucleotide-long RNAs. In contrast to [β-^{32}P]GTP-labeled RNAs, all of the label could be removed by phosphatase, demonstrating di- or triphosphate termini (data not shown). The approximately 47-nucleotide-long transcript probably corresponds to leader RNA (8). Testa et al. (30) reported short triphosphate-initiated RNAs from the beginning of the *N*, *NS*, and *M* genes which are 40, 28, and approximately 70 nucleotides long, respectively. The minor transcripts shown in Fig. 1, lane 4, above and below the leader RNA, are probably identical to these RNA species. Particularly, the transcripts which migrate slightly ahead of the xylene cyanol dye may represent the uncapped form of the 40-nucleotide-long transcript of the *N* gene shown in Fig. 1, lanes 2 and 3 (also see below). The abundant 11- to 14-nucleotide-long transcripts were recently shown by Pinney and Emerson (18) also to result from a high frequency of initiation at the beginning of the *N* gene. Although frequent initiation was observed in the present study, no capped transcripts were found in the same region of the polyacrylamide gel (Fig. 1, lane 3). The shortest capped RNA was 23 nucleotides long.

To identify large GTP-initiated transcripts which might be hidden under the vast majority of capped RNAs, transcription was carried out in the presence of γ-thio-GTP with internal [α-^{32}P]CTP label. GTP-initiated RNAs were selected on Hg-agarose columns, which bind message-size RNAs (Fig. 1, lane 5) (29). The predominant RNA species found was approximately 28 nucleotides long and was presumably identical to the [β-^{32}P]GTP-labeled 28′-mer RNA in Fig. 1 (lane 2). Although some large GTP-initiated RNA species were selected on Hg-agarose, their molar amounts compared to the 28-nucleotide-long RNA were very low. These message-size RNA species may be identical to those found by Rose (21) in vivo.

Nucleotide sequence analysis of capped and GTP-initiated RNAs. Individual capped RNA species (Fig. 1, lane 3), as well as mixtures of capped RNAs from sections of the polyacrylamide gel containing larger species (above the xylene cyanol dye and up to message length), were eluted and subjected to partial hydrolysis in formamide. The fragments were separated on a 20% polyacrylamide gel (Fig. 2A, lanes 1 through 8). Except for lane 8 and lanes 5 and 7, which represent mixtures of short capped RNA species (also containing N message species), the fragments in all other lanes lined up exactly, demonstrating that the RNAs were subsets of each other sharing the same 5′ terminus. The spacings between RNA bands, a characteristic of a particular nucleotide sequence, were identical. The larger spaces caused by the addition of G residues were as marked. The positions of the G residues in the sequence suggested that most of the RNAs were derived from N mRNA. The RNA fragments in lane 8 (from the approximately 32-nucleotide-long RNA in Fig. 1, lane 3),

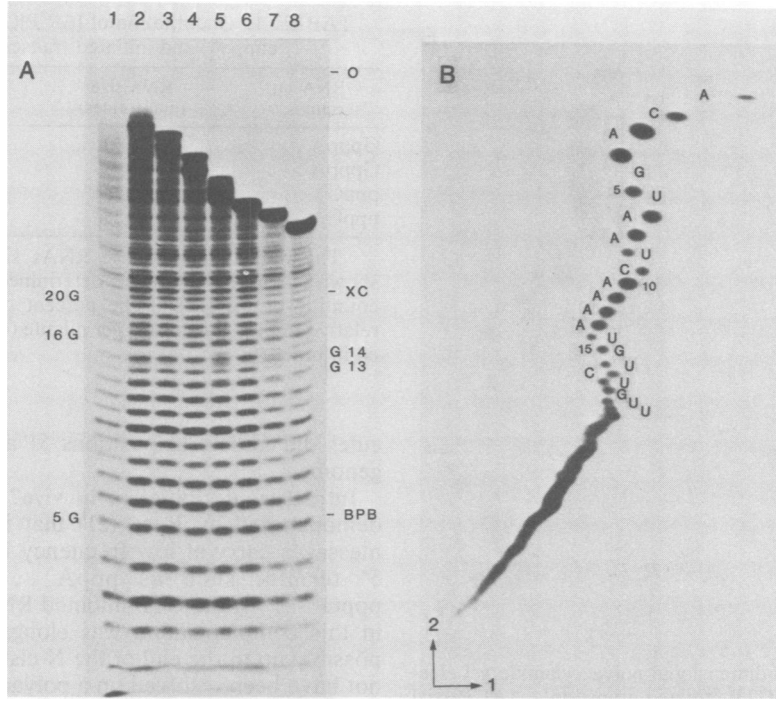

FIG. 2. Sequence analyses of the capped RNA species. [β-^{32}P]GTP-labeled capped transcripts, as shown in Fig. 1, lane 3, were partially hydrolyzed in formamide and separated on a 20% polyacrylamide gel (A). Lane 8, 32 N transcript; lane 7, 36 N transcript; lane 6, 40 N transcript. Lanes 1 through 5 contain mixtures of larger RNAs from gel sections above the xylene cyanol dye. (B) Two-dimensional separation of a partial hydrolysate of total capped RNAs.

however, showed a different pattern. This pattern was consistent with this RNA's being transcribed from the NS cistron (23). Two-dimensional sequence analysis of the total capped RNAs demonstrated that more than 90% of the RNAs share the 5′-terminal sequence of N message (Fig. 2B).

The nucleotide sequences of the prominent [β-^{32}P]GTP-initiated RNAs (28 and 24 nucleotides long; Fig. 1, lane 2) were analyzed in one and two dimensions. In addition, transcripts synthesized in the presence of γ-thio-GTP were selected on Affi-Gel 501 and labeled at their 3′ termini with [^{32}P]pCp and RNA ligase. A number of bands were detected, with a predominant 24-nucleotide-long RNA species (Fig. 1, lane 6). These RNA species could be reselected by using Affi-Gel 501, demonstrating that the transcripts retained intact γ-thio-GTP termini after ligation. Nearest-neighbor analyses of the 3′-terminally labeled RNAs, using RNase T_2, revealed the transfer of the label from p*Cp to the 3′-terminal bases as indicated in Fig. 1, lane 6. Two-dimensional sequence analyses of partial formamide hydrolysates of the 3′-terminally labeled 24-nucleotide-long RNA species (Fig. 3B) showed the same nucleotide sequence as partial RNase T_2 digests of the 28-nucleotide-long, 5′-terminally labeled transcript (Fig. 3A). G and U as well as A and C residues were also identified by partial enzymatic digestions (data not shown). Nearest-neighbor analyses together with the analyses of partial formamide hydrolysates suggested that the marked RNAs (Fig. 1, lane 6) were subsets of each other sharing the same 5′ terminus and differing by one nucleotide in length. The 3′-terminal nucleotides matched exactly the sequence of the 28′-mer from positions 20 to 28. RNA ligase preparations sometimes contain low amounts of 3′ exonuclease activities which, besides premature terminations, presumably generated the subsets of fragments (25).

The nucleotide sequence of the 28-mer and 24-mer GTP-initiated RNAs is indicated in Fig. 4. Both were specifically initiated at position 91 of the VSV genome, 41 nucleotides within the *N* gene. This initiation site represents not only the first example of an intracistronic initiation, but also the first GTP-initiated transcript in vitro. Interestingly, capped transcripts terminated preferentially at positions 86 to 90 of the genome adjacent to the new initiation site. Table 1 shows a quantitation of the capped and triphosphate-ending RNAs. As indicated, 12.1% of the total [β-^{32}P]GTP-labeled transcripts terminated at positions 86 to 90, preferentially at position 90

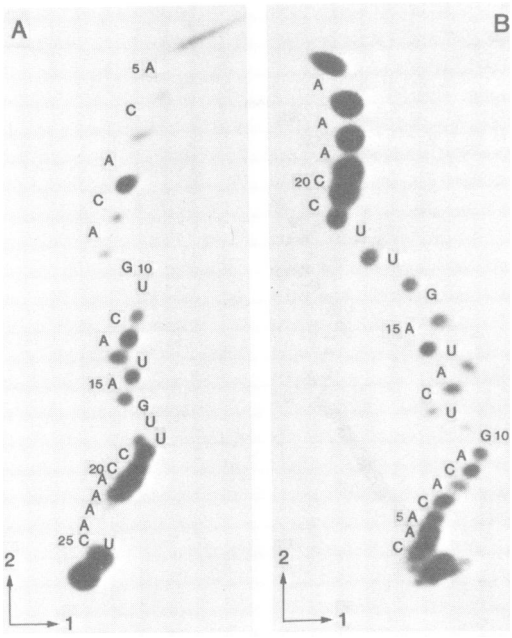

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of GTP-initiated transcripts. (A) Partial RNase T_2 digest of [β-^{32}P]GTP-labeled, 28-nucleotide-long transcript shown in Fig. 1, lane 2. (B) Partial hydrolysate of 3′-terminally labeled, 24-nucleotide-long transcript shown in Fig. 1, lane 6. Position 1 in the sequence designates the 5′ terminal GTP.

(7.8%), giving rise to 36 to 40-nucleotide-long, capped RNAs. Transcripts initiated with GTP at position 91 (7.6%) were 24 and 28 nucleotides long, preferentially 28 nucleotides long (5.6%). The smaller 10 to 23-nucleotide-long RNAs, together representing 3.1% of the labeled RNAs, were not further characterized. The termination of capped transcripts at position 90 and initiation at position 91 occurred at about the same frequency, suggesting that the same polymerase might have terminated and specifically reinitiated at this intracistronic site. However, it is not ruled out that two separate polymerase molecules did initiate at positions 51 and 91 of the genome.

TABLE 1. Quantitation of [β-^{32}P]GTP-labeled, capped, and initiated transcripts

RNA terminus	RNA size (nucleotides)	cpm[a]
GpppA . . .	>40	375,053 (77.2)
GpppA . . .	36–40	58,967 (12.1)
pppG . . .	24, 28	36,878 (7.6)
pppG . . .	10–23	14,829 (3.1)

[a] The radioactivity of the RNAs similar to those shown in Fig. 1, lane 2 was determined by Cerenkov counting of gel slices. The percent of radioactivity relative to the total counts per minute (cpm) is indicated in brackets.

Intracistronic initiation in vivo? It has been demonstrated by Rose (21) that in vivo VSV messages carry at low frequency heterogenous 5′ termini such as pppA . . . and also pppG . . . . If the GTP-initiated RNA described in this communication was elongated in vivo, possibly up to the end of the N cistron, it might not have been resolved on a polyacrylamide gel from normal N mRNA. To test for this possibility, RNAs from VSV-infected BHK-21 cells were isolated 3.5 h after infection and were separated on 10 to 30% sucrose gradients (Fig. 5). Adjacent fractions were combined into eight pools, and the RNAs were annealed to a single-stranded, 5′-terminally labeled cloned DNA which was identical to the first 214 nucleotides of VSV genomic RNA. The label was introduced by polynucleotide kinase at a position corresponding to the G residue in position 214 of VSV genomic RNA (11, 15). After annealing, the single-stranded tails were digested with nuclease S1 in high-ionic-strength buffer, and the resistant DNA-RNA duplex was separated on a 7.5% polyacrylamide gel next to terminally labeled double-stranded *Hae*III restriction fragments of ϕX174 DNA. A single band was detected in lanes 6 and 7 of Fig. 5, corresponding to duplex formation with 15 to 18S RNA, the size of N

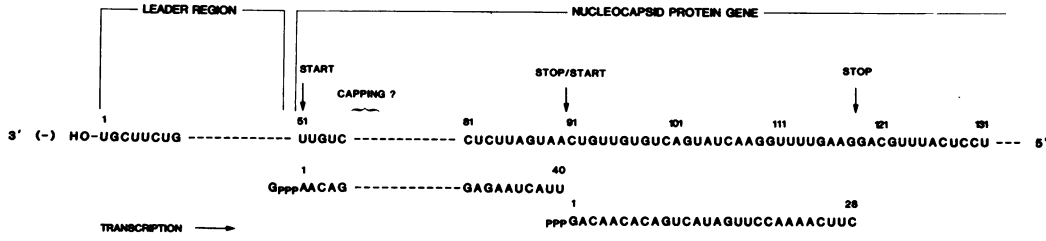

FIG. 4. Origins of the short capped and GTP-initiated in vitro transcripts. Short capped RNAs were preferentially 40 nucleotides long and were terminated at position 90 of the VSV genome. Uncapped, GTP-initiated transcripts started at position 91 and were terminated at position 118.

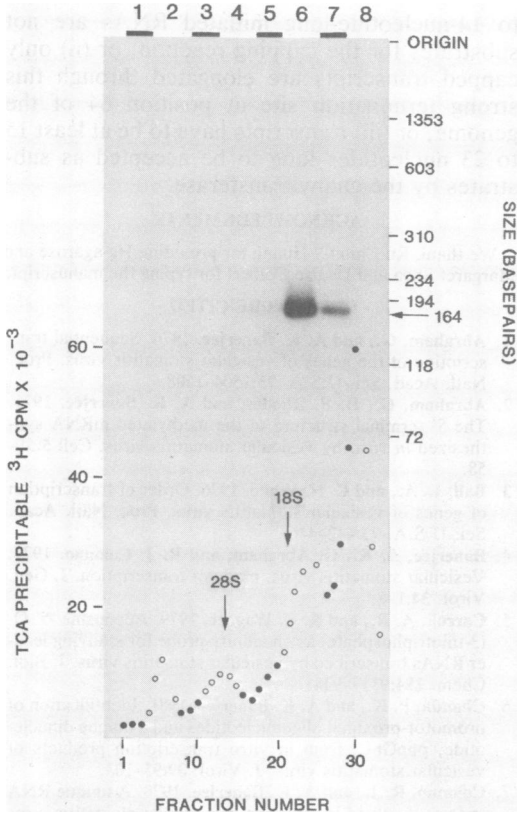

FIG. 5. Annealing of in vivo transcripts of VSV to a specific 5'-terminally labeled, single-stranded, cloned DNA. This probe was identical to the nucleotide sequence from positions 1 to 214 of the VSV genome. Intracellular transcripts from VSV-infected BHK-21 cells were separated on a 10 to 30% sucrose gradient. After fractionation, adjacent fractions were combined into eight pools as indicated (○, ●). The RNAs were annealed to the DNA probe. After nuclease S1 digestion, the resistant duplexes were separated on a 7.5% polyacrylamide gel next to *Hae*III restriction fragments of φX174 DNA. The sizes and positions of some of the restriction fragments on the gel were as indicated. The positions of cellular 28S and 18S rRNAs were determined by absorbancy at 260 nm.

mRNA. The size of the duplex was that expected for a duplex between N mRNA and the labeled DNA, approximately 164 base pairs. However, we did not observe a 124-base pair duplex that would be expected for a duplex between transcripts initiated at position 91 of the genome and the 5'-terminally labeled DNA probe. These data suggest that intracistronic initiation with GTP in vivo (3.5 h after infection) did not generate significant amounts of stable transcripts large enough to protect the terminally labeled probe. We estimate the level of detectability to be 5% of the amount of N message.

DISCUSSION

In this communication we have identified a new initiation site of VSV in vitro transcription. Initiation with GTP at this intracistronic site gave rise to short, predominantly 24- and 28-nucleotide-long transcripts. The minor portion of message-size GTP-initiated transcripts may be identical to those found by Rose (21) in vivo. Interestingly, initiation took place at the same position on the genome where the capped, 40-nucleotide-long *N* gene transcripts terminated. In addition, the frequencies of stop and start were almost the same within a factor of 2. We have not been able to demonstrate initiation with GTP at this intracistronic site when UTP was omitted from the transcription reaction. Under these conditions, frequent initiations did occur at the beginning of the *N* gene (6; Schubert and Lazzarini, unpublished data); however, we did not detect initiation with GTP resulting in a 10-nucleotide-long transcript, as expected from the sequence. Although we cannot rule out a very low frequency of GTP starts, these observations suggest that initiation at position 91 may be dependent on termination at position 90 and therefore might be sequential.

A comparison of the sequence of this initiation site with other initiation or resumption sites of VSV replication and transcription is shown in Fig. 6. The sites of initiation or resumption of RNA synthesis, as in the case of the fall-back sequence which is involved in the generation of a particular class of defective interfering particle RNAs (24), are as indicated by arrows. Initiations have been demonstrated for the (−) and (+) genomes (for review, see 18) as well as the *N*, *NS*, and *M* genes (24, 31), but not yet for the *G* and *L* genes. Although the sequences are not congruent, they all share the sequence 5′ . . . $^{G}_{C}U^{G}_{C}UU$ . . . 3′ in which the G and C residues can be exchanged, such as GUCUU, GUGUU, or CUGUU. Initiations take place, preferential-

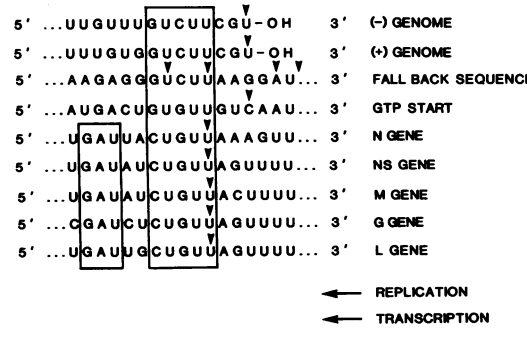

FIG. 6. Comparison of initiation sites of VSV replication and transcription. The sites of initiation and resumption of RNA synthesis are indicated by arrows.

ly using ATP, right at or a few nucleotides upstream from these sites. Obviously, short pentamers like these alone cannot guarantee specific initiation. Therefore, other structural features or factors must be involved to ensure specificity.

We have not detected in vivo initiation with GTP at this intracistronic site and elongation to yield a transcript of at least 124 nucleotides in length (Fig. 5). We estimate the level of detectibility of our assays at approximately 5% relative to the amount of N mRNA. Consequently, it is not clear whether in vivo initiation does occur at this site but only infrequently. Further investigations will require a more sensitive and more specific screening procedure. It is interesting that transcripts initiated at this site lack the ribosome binding site for N protein translation, but if elongated beyond position 127 of the genome could contain the reported minor ribosome binding site (22).

Testa et al. (30) reported short di- and triphosphate-terminating RNAs transcribed from the beginning of the *N* and *NS* genes, respectively. The transcripts described here were almost identical in size (36 to 40 and 32 nucleotides) but were capped. The role of these short cap, di- or triphosphate-containing RNAs is unclear. It was postulated that the di- or triphosphate-ending RNAs represent precursors for capped messages (30). Pulse-chase experiments, however, demonstrated that these stable transcripts were released from the template and were not chased into capped RNAs (16a). Premature termination of transcription occurs most often at position 64, only 14 nucleotides into the *N* gene, as recently demonstrated by Pinney and Emerson (18) (also see Fig. 1, lane 4). These authors suggested that premature termination at this site resulted from an interaction between the viral M protein with the transcriptive complex in vitro (19). Possibly this kind of interaction might also trigger termination at positions 90 and 118 after transcription of the capped 40-mer RNA or of the GTP-initiated RNA. Initiation with GTP at position 91 might merely be the result of this termination event. Termination in this case takes place in the proximity of a site which shares nucleotide similarities with other initiation sites (Fig. 6).

Initiation at the beginning of the *N* gene occurred very frequently and yielded short transcripts (Fig. 1, lane 4). Capped RNAs of the same size, however, were not detected (Fig. 1, lane 3). The shortest capped RNA observed was 23 nucleotides long. In addition, caps were not detected in transcription reactions lacking UTP, despite the fact that initiation at the *N* gene occurred frequently, yielding pppAACAG (6; Schubert and Lazzarini, unpublished data). These data suggest that either (i) these short 5- to 14-nucleotide-long initiated RNAs are not substrates for the capping reaction, or (ii) only capped transcripts are elongated through this strong termination site at position 64 of the genome, or (iii) transcripts have to be at least 15 to 23 nucleotides long to be accepted as substrates by the guanyltransferase.

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