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Initiation of RNA synthesis *in vitro* by vesicular stomatitis virus: single internal initiation in the presence of aurintricarboxylic acid and vanadyl ribonucleoside complexes

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ABSTRACT

In the presence of aurintricarboxylic acid (ATA) and vanadyl ribonucleoside complexes (VRC), we have isolated and characterized a small RNA, product of VSV in vitro transcription. This RNA is capped and lacks poly(A) at its 3'-end. Nucleotide sequence analysis revealed that this RNA corresponds to the 5'-terminal transcription product of the N-gene. The termination of the transcription occurs precisely at the 118th base from the 3'-end of the VSV genome. Analysis of the nucleotide sequence around this region reveals a potential secondary structure. Photoreaction of the VSV with 4'-substituted psoralen fails to inhibit the synthesis of the 68-mer RNA under conditions where full length mRNA synthesis is blocked, indicating that the psoralen binding site is located further into the N-gene. Since this RNA is the only in vitro transcription product synthesized under these conditions, the existence of two types of polymerase activities, one for the synthesis of leader RNA and one for mRNA, is suggested.

INTRODUCTION

The RNA genome of vesicular stomatitis virus (VSV) is transcribed in vitro by virion associated RNA polymerase into six discrete RNA species. These include the 47-nucleotide leader RNA and five capped and polyadenylated mRNAs encoding the viral proteins N, Ns, M, G and L (1). Hybridization studies and UV light inactivation studies have demonstrated that transcription is sequential along the genome in the order 3'-leader N-Ns-M-G-L-5' (2,3). Three general models of transcription have been proposed to account for the sequential and polar nature of VSV transcription. In the cleavage model the monocistronic mRNAs are generated via cleavage and processing of a precursor RNA (4,5). In the stop-start model the mRNA is generated by termination and reinitiation of transcription at each intercistronic region (6,24). The third multiple initiation model -- which is based on observation that in addition to leader RNA at least three promotor-proximal RNAs can be detected almost immediately after activation of the virion polymerase in vitro -- suggested that initiation of transcription occurs simultaneously at the beginning of each gene (7). However,

these short triphosphate-containing oligonucleotides are found in very low amounts and are not precursors to mature viral mRNAs. Recently, Schubert et al. (1982) found VSV in vitro transcription products that were GTP initiated intracistronically on the VSV genome (8), and Pinney and Emersson have observed in vitro synthesis of oligonucleotides 11 to 14 bases long, representing uncapped 5'-sequence of N-gene mRNA (9). From these results it seems clear that the VSV polymerase can initiate several RNA chains in addition to the leader RNA; however, such transcripts represent a small portion of the total RNA synthesis products, and therefore it is not clear if such initiation is relevant to the normal transcription process. In an effort to provide more conclusive evidence for either of the models described above, we have used RNase inhibitors ATA and VRC in our in vitro transcription reactions.

In this communication we report evidence of a single RNA transcript initiated internally in the presence of the RNase inhibitors aurintricarboxylic acid (ATA) and vanadyl ribonucleoside complexes (VRC). This RNA product corresponds to the 5'-terminal 68 nucleotides of the N-mRNA. Since this RNA is the only in vitro transcription product synthesized under these conditions, the presence of two types of polymerase activities for the synthesis of leader RNA and for mRNA are suggested.

MATERIALS AND METHODS

Materials:

RNases T_1, Phy M, B. cereus, and U_2 were purchased from PL-Biochemicals and used as described. Bacterial alkaline phosphatase and calf intestinal phosphatase were purchased from Boehringer Manheim Corp., Indianapolis, Indiana. [α^{32}P]GTP and ^{32}Pi were purchased from Amersham Corp., Chicago, Illinois. Unlabelled triphosphates were obtained from ICN, California. AMT was obtained from HRI Associates, Emeryville, California; VRC from BRL, Maryland, and ATA from Sigma Chemical Co., St. Louis, Missouri.

Virus growth and in vitro transcription:

BHK-21 cells, adapted to suspension culture, were used for virus growth. The details of infection and virus purification have been described (10). In vitro transcription reactions were identical to those described previously (11). The transcripts were labelled with either [α^{32}P]GTP or [β^{32}P]GTP; the latter was prepared with 10 mCi of ^{32}P Pi using the procedure by Kaufman et al.(12). The RNA products were purified

by phenol extraction and separated from unincorporated nucleotides by spincolumn chromatography on Biorad P-6. The labelled RNA's were separated on 6% polyacrylamide gels.

Nucleotide sequence analysis:

[5'-^{32}P]-labelled small RNA was sequenced enzymatically (13). Digestions were carried out at 55°C for 15 min in 10 µl which contained 20 mM sodium acetate, 1 mM EDTA and 2 µg carrier tRNA. In addition, the RNase reactions with enzymes T_1 (G-specific), U_2 (A-specific) and Phy M (A- and U-specific) were carried out in 7 M urea while that with B. cereus (U- and C-specific) required no urea. The U_2 reaction was buffered at pH 3.5 and the others at pH 5.0. After reaction, the samples were run on 8% polyacrylamide gels and autoradiographed.

Photochemical reaction with AMT:

Ultraviolet irradiation of low salt RNPs was carried out at 0°C in 10 mM Tris-HCl (pH 8.0). The samples to be irradiated were put in Eppendorf tubes partially immersed in a 0°C water bath. The bath was surrounded by a double-walled glass vessel containing a circulating 40% (w/w) cobaltous nitrate solution. The solution served as a temperature regulator and an ultraviolet and visible light filter which had a maximum transmittance at 365 nm light (window: 320-380 nm). The samples were irradiated for different periods of time, indicated in figure legends, by two GE 400 W mercury lamps, one on each side of the sample. The intensity of light at the sample was approximately 100 mW/cm^2.

RESULTS

Detection of a small RNA in the presence of VRC and ATA:

In vitro RNA synthesis by triton-disrupted virus was carried out in the presence of various concentrations of VRC. As shown in Fig. 1a, increasing concentrations of VRC resulted in progressive inhibition of both mRNA and leader RNA synthesis; however a small RNA was synthesized even at the highest VRC concentration (1 mM) where all other RNA synthesis was completely inhibited. Since VRC is an RNase inhibitor we investigated the effect of other RNase inhibitors on *in vitro* transcription of VSV. Fig. 1b shows the effect of increasing concentrations of ATA on RNA synthesis. Like VRC, mRNA synthesis and leader RNA synthesis was inhibited; however, the same small RNA was synthesized even at the highest ATA concentration.

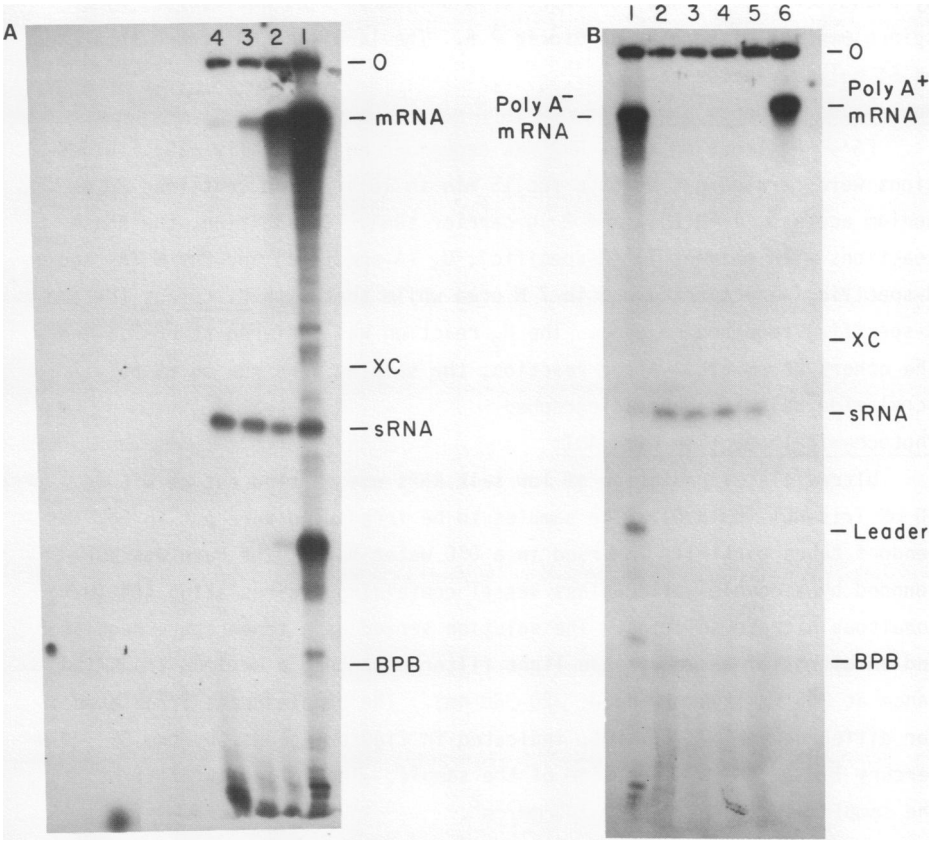

Fig. 1a. Analysis of in vitro transcription products of VSV in presence and absence of VRC. Transcription by triton-disrupted VSV was carried out in 0.2 ml standard reaction mixture (11) containing 20 μ Ci [α^{32}P]GTP for 1 hr at 30°C. VRC was added at different concentrations. The product RNA was purified as described in Materials and Methods and analyzed by 6% polyacrylamide gel electrophoresis. Lane 1: Without VRC. Lane 2: With 0.2 mM VRC. Lane 3: With 0.5 mM VRC. Lane 4: 1.0 mM VRC. The positions of the dyes xylene cyanol (XC) and bromophenol blue (BRB) were as indicated. O, origin.

Fig. 1b. Analysis of in vitro transcription products of VSV in the presence and absence of ATA. Transcription conditions were identical to Fig. 1a. ATA was added at different concentrations. The product RNA was chromatographed on oligo(dT)-cellulose column and the polyA$^-$ and polyA$^+$ fractions were separated on 6% acrylamide gel. Lane 1 through Lane 5 -- PolyA$^-$/RNA. Lane 1: Without ATA. Lane 2: 0.2 mM ATA. Lane 3: 0.4 mM ATA. Lane 4: 0.8 mM ATA. Lane 5: 1.0 mM ATA. Lane 6: Without ATA, polyA$^+$RNA. O, origin.

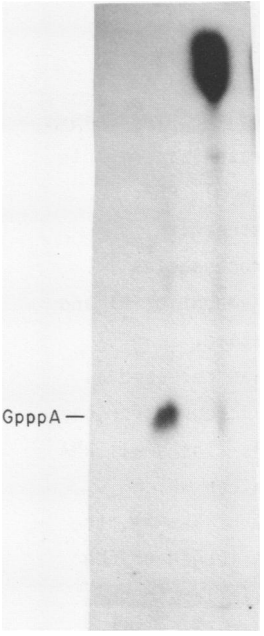

Fig. 2. Cap analysis of in vitro transcription product of VSV in the presence of 1 mM VRC. Transcripts labelled with [β^{32}P]GTP were completely digested using nuclease P_1 and phosphatase. The products were separated by high voltage paper electrophoresis at pH 3.5. Lane 1, Lane 2: The putative GpppA spot was eluted with triethylamine bicarbonate and digested with snake venom phosphodiesterase and phosphatase.

5'-terminal structure and chain length analysis of the small RNA:

The only RNA product synthesized during the in vitro VSV transcription in the presence of VRC and ATA was purified and passed through an oligo-dT cellulose column. Since it failed to bind to oligo-dT cellulose, this small RNA lacks a poly(A) tail on its 3' end. On the basis of its migration in a 6% polyacrylamide gel, its size appears to be approximately 60-70 bases long (Fig. 1a,b). To establish whether this RNA is a read-through leader RNA or an internally initiated RNA, we analyzed the 5'-terminal structure. The small RNA was terminally labelled by conducting the transcription in the presence of 1mM VRC and [β^{32}P]GTP. Unlike most other viral and eukaryotic mRNA, VSV messages conserve the beta phosphate of GTP in the cap (14). Therefore, labelling of the transcripts with [β^{32}P]GTP specifically identifies capped or GTP initiated RNA. Since leader RNA is not capped, the labelling of the small RNA with [β^{32}P]GTP indicates that it is an internally initiated RNA. Purified [β^{32}P]-labelled small RNA was digested with nuclease P_1 and calf intestinal alkaline phosphatase, and the product was analyzed by high voltage paper electrophoresis. A distinct band representing the cap structure migrated characteristically near GMP and migrated identically with authentic cap

marker 7mGpppA (Fig. 2). Radioactivity in the cap was released to Pi when the sample was digested with snake venom phosphodiesterase and phosphatase, confirming that the small RNA contained a 5'-terminal cap structure G(5')ppp(5')A. Since there are several reports of the synthesis of incomplete N-mRNA it was presumed that this RNA may represent incomplete N-mRNA (9,17,18). For determining the chain length of the small RNA, purified [β^{32}P]GMP labelled N-mRNA and small RNA were digested with nuclease P_1 and calf intestinal alkaline phosphatase and processed as described above. The chain length was calculated from the amount of Pi and GpppA released after digestion with nuclease P_1 and phosphatase. As shown in Table I, 0.34% of the radioactivity in N-mRNA was released as cap, which was close to the theoretical value of 0.35% and accounts for one cap per 285 G residues in a total chain length of 1325 (15). The small RNA on the other hand released 10.9% of the total counts as the cap which accounted for nine G residues. From the published sequence of N-mRNA, the ninth G residue is located at position 71. Thus, the chain length of the small RNA appears to be close to 70 bases. The number is similar to the estimated chain length determined from the gel electrophoresis.

Nucleotide sequence analysis:

To directly ascertain whether the small RNA indeed represents the 5'-terminal portion of N-mRNA, partial sequencing of sRNA was carried out. 5'-labelled small RNA was synthesized by *in vitro* transcription in the

Table I. Determination of Chain Length of Small RNA from CAP Analysis

RNA Species	cpm in Gp*ppA	cpm in Pi*	Percent CAP	No. of G Residues	Chain Length
NmRNA	1295	374500	0.34	289	(1325)[a]
sRNA	24050	195870	10.9	9	(71)[b]

[β^{32}P]GTP-labelled RNA was synthesized in *in vitro* transcription mixture after incubation for 4 hrs at 30°C. sRNA was purified by 6% polyacrylamide gel electrophoresis and N-mRNA from velocity-gradient. Aliquots of each RNA were digested with nuclease P_1 and calf intestinal alkaline phosphatase. The digest was analyzed by high voltage paper electrophoresis at pH 3.5. The radioactivity migrating at the positions of CAP and Pi was eluted and counted.

a. (1325) - Actual no. of G residues and chain length of N-mRNA are taken from Gallione, *et al.* (1981).

b. (71) - The chain length of sRNA was determined from the position of G residue no. 9 in the N-mRNA sequence of Gallione, *et al.*

presence of [β^{32}P]GTP. Purified small RNA was then partially digested with base specific RNases and digests were analyzed on an 8% gel. The sequence can be read directly from the autoradiogram. As shown in Fig. 3, the 3'-terminal 19 bases correspond to the predicted 3'-terminal portion of a 68-base-long 5'-terminal N-mRNA. These results prove that this small RNA is the first 68 nucleotides of the 5'-terminus of N-mRNA.

Synthesis of small RNA by psoralen-photoreacted VSV cores:

Talib and Banerjee (11) have recently shown that photoreaction with 4'-substituted psoralen modifies the VSV genome RNA and, as a consequence, inhibits mRNA synthesis in vitro by virion associated RNA polymerase. Moreover, it was found that photoreaction occurred within the N-gene near the 3' end of the viral genome. Since the small RNA synthesized in the presence of ATA and VRC appears to be the 5' terminal portion of N-mRNA, it

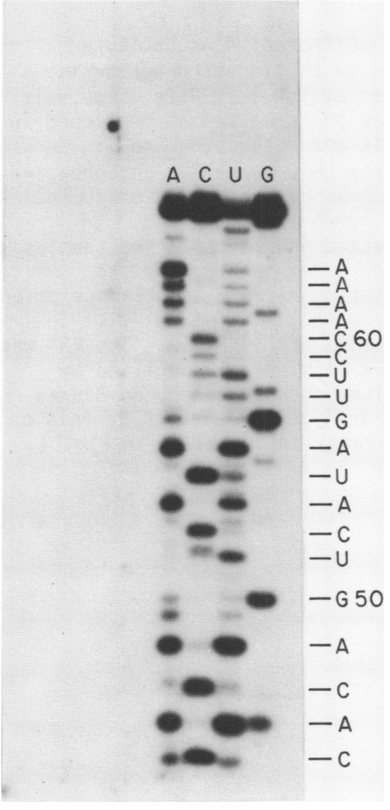

Fig. 3. Partial sequence analysis of 5'-^{32}P labeled 68-mer. [β^{32}P]GTP- labelled transcripts, as shown in Fig. 2, were partially digested with the following enzymes and the digests were analyzed on an 8% gel. Lane 1: RNase U_2 (A-specific). Lane 2: RNase B. cereus. Lane 3: RNase Phy M (U+ A specific). Lane 4: RNase T_1 (G-specific). Nucleotide sequence indicated is consistent with 3'-19 bases of 68-mer.

was of interest to study the effect of 4'-substituted psoralen on the synthesis of this RNA in vitro. Purified RNP was photoreacted with AMT in the presence of UV light for different time periods and the RNA synthesis was carried out in the presence of ATA. The products were analyzed by polyacrylamide gel electrophoresis. As judged by TCA precipitated counts, total RNA synthesis in the absense of ATA decreased more than 80% after 5 min. of photoreaction. As shown in Fig. 4, the synthesis of the small RNA remained virtually unchanged. These results indicate that the psoralen binding site on the genome RNA is beyond the termination site of the small RNA, which is 68 bases into the N-gene.

DISCUSSION

In this communication we have identified and characterized a small RNA synthesized by a single internal initiation. This RNA is synthesized in

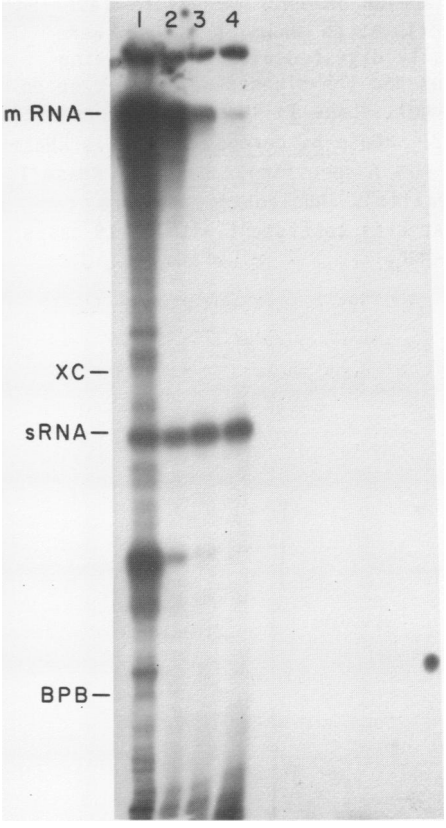

Fig. 4. Effect of 4'-substituted psoralen on the in vitro synthesis of 68-mer by low salt RNPs. Low salt RNPs were photoreacted as mentioned in Materials and Methods. Lane 1: unphotoreacted. Lane 2: photoreacted 1 min. Lane 3: photoreacted 2 min. Lane 4: photoreacted 5 min. Unphotoreacted and photoreacted samples were then added in the standard transcription mixture and transcription was carried out at 30°C for 2 hr in the presence of [α^{32}P]GTP. The RNA was extracted with phenol, purified by spin-column chromatography on Biorad P-6 and analyzed by electrophoresis on a 6% polyacrylamide gel as in Fig. 1.

the presence of RNase inhibitors VRC and ATA, which are known to inhibit RNases as well as free polymerase (16). Our results confirm the earlier observations of Hunt and Wagner (26) that ATA inhibits VSV transcription in vitro; however since they analyzed the in vitro transcription products by TCA precipitation, they were unable to detect the small RNA reported here. With both of these inhibitors only one RNA transcript is synthesized. This RNA contains the normal VSV cap at its 5'-end but lacks poly(A) at its 3'-terminus. From the analysis of the cap (Table I) and partial RNA sequencing it was found that the RNA represents the 5'-terminal 68 nucleotides of N-mRNA. Since the only RNA transcript synthesized under these conditions is the 68 mer, it is unlikely that this RNA is generated by nonspecific degradation of N-mRNA by nucleases. It appears that the synthesis of the small RNA results from an authentic internal initiation event by VSV transcriptase. The molar ratio of 68 mer RNA synthesis relative to the synthesis of leader RNA under control conditions is 1:1. It is interesting to note that the results of Iverson and Rose (17) show greater synthesis of the 5'-proximal sequences of N-mRNA both in vitro and in vivo than of the 5'-proximal sequences of the other mRNA's. Moreover, UV-irradiated VSV also preferentially synthesizes the 5'-terminal sequences of N-mRNA (17). These results and those of others (9,18) indicate an internal initiation of RNA synthesis at the beginning of the N gene. The small RNA reported here seems to terminate precisely at the 118th base from the 3'-end of the VSV genome. Termination of a GTP initiated transcript at the same site was also observed by Shubert et al. (8). Analysis of the nucleotide sequence around this region reveals a potential secondary structure (Fig. 5). It has been suggested that

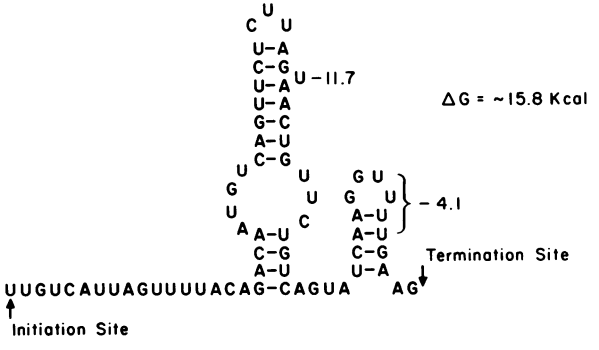

Fig. 5. Secondary structure of the termination region.

stem-and-loop structures cause retardation of polymerase movement through termination regions, whereas uridine residues facilitate the release of transcripts (23). Hairpin induced pauses of polymerases on the template strand have been shown to occur in the αβ replicase system (19) and in the Trp operon in E. coli (20). Recently Aloni et al. (21) have reported secondary structure dependent transcription and proposed that an attenuation mechanism resembling that found in prokaryotes regulates SV40 late transcription. It thus appears that structure induced pauses or terminations may be a general phenomenon of eukaryotic as well as prokaryotic gene regulation.

Model of Transcription:

One interesting aspect of this study is that it shows for the first time that VSV polymerase can exclusively initiate internally at the N-gene without the synthesis of leader RNA and other promotor proximal sequences. This result suggests a single initiation mechanism. A recent report by Iverson and Rose (17) of sequential synthesis of 5'-proximal VSV mRNA sequences also does not support the multiple initiation model. Recently Emerson (24) has proposed, on the basis of partial transcription with virions and reconstituted cores, a single initiation model for VSV transcription. Results presented in this paper do not support this hypothesis. The reasons for this contradiction are not clear at present but it is important to point out that complete and partial transcription do not represent similar molecular events; for example, complete transcription at low salt concentrations (0.07 - 0.005 M) favors internal initiation (25), that is, an increase in N-gene transcript relative to leader RNA. However, during partial transcription, low salt concentration favors 3'-end initiation, that is, increased synthesis of the leader relative to the mRNA oligonucleotides (24). This discrepancy indicates differences in complete and partial transcription. Moreover, it is still unclear whether partially initiated oligonucleotides are precursors to mature transcripts. Previous attempts by us and by others have failed to chase them into mature transcripts (26, 27). The differential inhibition of leader relative to N-mRNA proximal sequence by ATA and VRC suggests to us that perhaps two types of polymerase activities are involved in VSV transcription: one which is involved in the synthesis of a short uncapped unpolyadenylated 47 nucleotide leader RNA and is sensitive to VRC and ATA; and the other which is involved in initiation of the synthesis of capped and polyadenylated mRNAs and is less sensitive to these inhibitors. If true, overall VSV trans-

cription may be regulated by two activities, one initiating at the 3'-end of the genome giving rise to leader RNA, and the other responsible for initiation of mRNA synthesis, which starts by internal initiation 51 nucleotides from the 3'-end. The five messenger RNA's are most probably made by the latter activity employing a stop-start mechanism. Which of the two proposed activities is essential for completion of the synthesis of the mRNA's is not clear. It should be noted that in the case of SV40 transcription, it has been proposed that RNA polymerase may function in two forms, the 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) resistant form, which initiates transcription, and the sensitive form, which elongates transcription (22). Further experiments are needed to elucidate the exact mechanism of ATA and VRC inhibition of RNA synthesis.

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