Translation of an Uncapped mRNA Involves Scanning*

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*tat, an essential gene of human immunodeficiency virus, when placed under the control of the RNA polymerase III promoter from the adenovirus VA RNA1 gene, is transcribed into an uncapped and nonpolyadenylated mRNA. This VA-Tat RNA is translated to produce functional Tat protein in transfected mammalian cells (Gunnerỹ, S., and Mathews, M. B. (1995) Mol. Cell. Biol. 15, 3597–3607). The presence of an upstream open reading frame (ORF) in VA-Tat RNA is inhibitory to the translation of the Tat ORF, suggesting that the RNA is scanned during translation even though it is uncapped. Because the effect of the upstream ORF is relatively small (about 2-fold), we sought more definitive evidence of scanning by introducing secondary structures of varying stabilities into the 5′-untranslated region of VA-Tat RNA. The results of transfection experiments showed that highly stable secondary structure was inhibitory to Tat synthesis, whereas structures of lower stability were not inhibitory, confirming that uncapped mRNA is subject to scanning. Furthermore, translation of the downstream ORF was reduced but not eliminated by mutations that caused the upstream ORF to overlap the Tat ORF. Extending the overlap of the two ORFs further decreased the translation of the downstream ORF. This observation implies that ribosomes reinitiate after termination, possibly after migrating in a 3′ to 5′ direction through the overlap region of the mRNA. Similar results were obtained with a capped polymerase II transcript, indicating that the translation of polymerase II and polymerase III transcripts occurs through similar mechanisms.

All known eukaryotic cellular mRNAs are capped, and most of them are functionally monochronic with initiation of translation taking place at the 5′ proximal AUG codon. These features are consistent with the scanning model of translational initiation (1). According to this model, cap-binding initiation factors bind to the 5′ cap structure of the mRNA and facilitate assembly of the 40 S initiation complex. The complex then migrates along the 5′-untranslated region (5′-UTR) of the RNA until it encounters the first initiation codon, AUG, present in a favorable sequence context, whereupon it binds the 60 S ribosomal subunit to complete the 80 S initiation complex (1). Translation then proceeds into the elongation phase. A recent reinterpretation of the findings proposes that an initiation factor complex, rather than the 40 S ribosomal subunit, scans the 5′-UTR, causing unwinding of the RNA, which then facilitates ribosome binding (2). According to this alternative model, the complex can bind randomly along the RNA but binds to the 5′ cap most efficiently (3). Exceptions to the “first AUG” rule were initially attributed to leaky scanning, but it is now clear that internal initiation and reinitiation can occur. Picornaviral RNAs and some cellular mRNAs contain long 5′-UTRs with several AUUGs upstream of the authentic initiator codon. These RNAs are also furnished with an internal ribosome entry site that allows ribosomes to bind internally (3). The internal ribosome entry site element confers cap-independent translation upon the mRNA and plays a role comparable to that of the cap structure in binding the 40 S ribosomal subunit to the mRNA for translation. The translation of mRNAs containing upstream open reading frames (ORFs) has been thoroughly studied in the yeast GCN4 gene (4). In this case, it has been proposed that the ribosome, after translating an upstream ORF, may retain competence to reinitiate at the downstream ORF.

The efficiency of mRNA translation is dependent on several features including the local sequence context surrounding the initiation codon (5). In accordance with the scanning model, very stable secondary structures in the 5′-UTR are inhibitory to translation, presumably because they obstruct the movement of the scanning moiety along the 5′-UTR (5). Structural elements of low stability are apparently readily penetrated and do not have any effect on the translation of the mRNA. On the other hand, a stable structure in the coding region does not inhibit the progress of elongating 80 S ribosomes (1). The presence of an upstream ORF in its 5′-UTR is another feature that can also affect the translational efficiency of the main open reading frame of an mRNA (6). In conformity with the scanning model, the upstream ORF generally exerts a negative effect on the translation of a downstream ORF. Reinitiation at the downstream ORF after translation of the upstream ORF has also been observed (7, 8). In instances where the two ORFs overlap, it has been proposed that ribosomes terminate at the end of the upstream ORF then migrate backward to the initiation codon of the downstream ORF where they reinitiate (9–11). This 3′ to 5′ movement, here called “backscanning,” may not be universal, since translation of some downstream ORFs is abolished by the introduction of an overlapping upstream ORF (12–14). An alternative explanation for this phenomenon is that ribosomes paused at the termination codon of the upstream ORF may slow the progress of following ribosomes that have bypassed the upstream AUG, thereby facilitating their initiation at the downstream AUG.

Recently, we reported the translation in vivo of an RNA polymerase III (pol III) product that is an uncapped and nonpolyadenylated mRNA (15). To generate this RNA, we placed

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1 The abbreviations used are: 5′-UTR, 5′-untranslated region; ORF, open reading frame; pol, polymerase; HIV, human immunodeficiency virus; sl, stem and loop; nt, nucleotide(s); S, stop codon; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus.
the human immunodeficiency virus-1 (HIV-1) Tat protein ORF under the control of the adenovirus VA RNA_{9} gene promoter, a strong pol III promoter. The resultant construct, pVA-Tat, synthesizes VA-Tat RNA that is transported into the cytoplasm and recruited for translation, resulting in the production of functional Tat protein in transfected cells. The translation of the pol III transcript is inefficient compared with that of a Tat transcript synthesized by pol II, which is capped and presumably polyadenylated. These findings indicate that neither the cap structure at the 5’S end nor the poly(A) tail at the 3’ end of an mRNA is essential for translation, although these features may be stimulatory.

To gain insight into the mechanism of translation of an uncapped mRNA, we examined the effects of alterations in the structure of VA-Tat RNA. VA-Tat RNA contains a short upstream ORF that is out-of-frame with the downstream Tat ORF. Incapacitation of the upstream ORF resulted in a small increase in translation of the downstream Tat ORF, raising the possibility that the RNA is scanned (15). As a critical test of this inference, we inserted secondary structures into the 5’-UTR of VA-Tat RNA and also made point mutations that caused the two ORFs to overlap. The results of transient expression assays confirm that the RNA is scanned for translational initiation despite being uncapped. Furthermore, the data are suggestive of reinitiation at the downstream AUG after termination of translation of the upstream ORF, possibly implying that ribosomes can backscan over the short distance of the overlap sequence.

**EXPERIMENTAL PROCEDURES**

**Construction of Stem and Loop (s) Mutants—**A BamHI restriction enzyme site was created at +72 of pVA-Tat by site-directed mutagenesis to produce pVT.Bam. For the construction of pVT.s1.30 and pVT.60, two oligonucleotides were synthesized, one identical to and another complementary to the sequence between nt +76 and +112 of pVA-Tat (AGCTTCGACATAGCAGAATAGGCGTTACTCGACAGAG). Both oligonucleotides carried four extra nucleotides at the 5’t end with respect to the transcription start site (Fig. 2A). The plasmid pVT.s1.30 contained 22 nt (nt +76 to +97 with respect to the transcription start site) inserted in VT.s1.30 RNA and pVT.30 were similarly constructed using oligonucleotides that correspond to nt +76 to +97 of pVA-Tat. The upstream ORF was incapacitated in all these constructs by mutating the upstream AUG (nt +44 to +46) to GCG. The translational activities of these transcripts were tested in an HIV-LTR transactivation assay, which takes advantage of the fact that HIV-1 Tat protein stimulates transcription from the HIV-LTR (21). pVA-Tat or derivatives thereof were transfected with the reporter construct pHIV-CAT, which contains the HIV-LTR driving the CAT reporter gene. The expression of Tat protein was monitored by measuring the increase in CAT activity in cells cotransfected with Tat-producing plasmids as compared with basal CAT activity in cells transfected with pHIV-CAT only. Fig. 1B shows the results of such an assay performed using the stem and loop insertion mutants of pVA-Tat. Cells transfected with parental pVA-Tat.I produced 20–60-fold higher levels of CAT enzyme activity than cells transfected with pHIV-CAT only. For purposes of comparison, the level of transactivation given by the parental Tat plasmid was assigned a value of 100. Cells transfected with control constructs (pVT.30 and pVT.60) that were not predicted to contain secondary structure in their 5‘-UTR produced levels of CAT activity about 20% lower than that of pVA-Tat.I. The increased length of the 5‘-UTR probably accounts for this small decrease in activity. Cells transfected with pVT.s1.30 exhibited similar CAT enzyme activity, whereas transfection by pVT.s1.60, which encodes a structure of higher stability (ΔG = –60.5 kcal/mol), was 5-fold lower than that given by the parental plasmid. Northern blot analysis using a probe complementary to the 5‘ end of VA RNA verified that similar levels of RNA

**RESULTS AND DISCUSSION**

We showed previously that an RNA polymerase III product that is uncapped and nonpolyadenylated can serve as an effective mRNA in mammalian cells (15). The HIV-1 Tat protein ORF was placed under the control of the strong pol III promoter from the adenovirus VA RNA_{9} gene to construct pVA-Tat. In transfected HeLa cells, pVA-Tat is transcribed to produce VA-Tat RNA, which is transported into the cytoplasm and recruited for translation by ribosomes, resulting in the production of functional Tat protein. This indicated that neither the cap structure at the 5‘ end nor the poly(A) tail at the 3‘ end of an mRNA is essential for translation.

The VA-Tat transcript is 368 nt long and contains two out-of-frame ORFs: the first ORF is 45 nt long and lies between nucleotides 44 and 88, whereas the second, which encodes Tat, lies between nucleotides 124 and 339 and is 216 nt long. The two ORFs are separated by 37 nt, and both have start codons in good sequence contexts for translational initiation (AUCUAG-GCG and GAAAUUGGAG, respectively). Mutation of the upstream AUG to GCG (in construct pVA-Tat.I), effectively eliminating the upstream ORF, increases synthesis of Tat from the downstream ORF by about 2-fold (15). This inhibitory effect of the upstream AUG raised the possibility that the RNA is scanned. To examine this possibility, we investigated the effect of introducing secondary structure into the 5‘-UTR of VA-Tat.I RNA. Stable secondary structure in the 5‘-UTR is envisaged to obstruct the movement of ribosomes or other moieties that are scanning the RNA from the 5‘ end, preventing their access to the start codon. Therefore, the inhibitory effect of secondary structure in the 5‘-UTR on the translation of an mRNA provides evidence for scanning (1). To create secondary structure in the 5‘-UTR of VA-Tat.I RNA, a stretch of sequence from the 5‘-UTR was duplicated in an inverted orientation such that the pairing of the two sequences forms a stem structure topped by a 4-nucleotide loop (Fig. 1A). The plasmid pVT.s1.30 contained 22 nt (nt +76 to +97) inserted at the same position. The predicted stability of the stem and loop structures in VT.s1.30 RNA and VT.s1.60 RNA was –31 and –60.5 kcal, respectively. Two control plasmids, pVT.30 and pVT.60, were also constructed that contained the equivalent sequences inserted in the sense orientation; transcripts of these plasmids were not predicted to form stable secondary structures in the 5‘-UTR.

The translational activities of these transcripts were tested in an HIV-LTR transactivation assay, which takes advantage of the fact that HIV-1 Tat protein stimulates transcription from the HIV-LTR (21). pVA-Tat or derivatives thereof were transfected with the reporter construct pHIV-CAT, which contains the HIV-LTR driving the CAT reporter gene. The expression of Tat protein was monitored by measuring the increase in CAT activity in cells cotransfected with Tat-producing plasmids as compared with basal CAT activity in cells transfected with pHIV-CAT only. Fig. 1B shows the results of such an assay performed using the stem and loop insertion mutants of pVA-Tat. Cells transfected with parental pVA-Tat.I produced 20–60-fold higher levels of CAT enzyme activity than cells transfected with pHIV-CAT only. For purposes of comparison, the level of transactivation given by the parental Tat plasmid was assigned a value of 100. Cells transfected with control constructs (pVT.30 and pVT.60) that were not predicted to contain secondary structure in their 5‘-UTR produced levels of CAT activity about 20% lower than that of pVA-Tat.I. The increased length of the 5‘-UTR probably accounts for this small decrease in activity. Cells transfected with pVT.s1.30 exhibited similar CAT enzyme activity, whereas transfection by pVT.s1.60, which encodes a structure of higher stability (ΔG = –60.5 kcal/mol), was 5-fold lower than that given by the parental plasmid. Northern blot analysis using a probe complementary to the 5‘ end of VA RNA verified that similar levels of RNA
were produced by pVA-Tat.I, pVT30, and pVT60, but pVTsl30 and pVTsl60 transcripts were not detected (data not shown), presumably because their secondary structures interfere. It is unlikely that these transcripts are degraded, however, because secondary structure generally increases RNA stability, and pVTsl30 expresses nearly as much transactivation activity as the parent plasmid, pVA-Tat.I.

These results resemble those reported for a preproinsulin mRNA that is transcribed by pol II (1), indicating that VA-Tat RNA is indeed scanned during translation despite its uncapped status. The scanning mechanism seems able to unwind secondary structures of low stability (ΔG = −30 kcal/mol) but is blocked by more stable structures (ΔG = −60 kcal/mol) in an uncapped mRNA as in capped mRNA (1). Furthermore, the decrease in activity accompanying the increased length of the 5′-UTR in VT.30 and VT.60 and VT.sl.30 is similar to that observed for capped mRNAs and is consistent with ribosome scanning. Our results indicate that the scanning process does not require the 5′ cap structure, but they do not necessarily exclude a role for the cap-binding initiation factor, eIF4F, and associated factors. Conceivably, such factors are recruited by uncapped mRNA, albeit inefficiently, by a cap-independent mechanism.

Given that VA-Tat RNA is scanned, it is not surprising that mutation of the termination codon of the upstream ORF of VA-Tat RNA, causing the two ORFs to overlap by 10 nt, leads to a decrease in transactivation activity (Ref. 15; Fig. 2B). The magnitude of the decrease (about 2-fold) is relatively slight, however, prompting us to consider possible explanations for the small effect. First, the start codon of upstream ORF may be leaky, i.e. not recognized by ribosomes despite its good context, so the ribosomes proceed to scan to the Tat AUG. Second, as VA-Tat RNA lacks a cap structure at its 5′ end, ribosomes might bind to VA-Tat RNA randomly and then commence scanning; those ribosomes binding between the two AUGs should not be affected by the overlap of the two ORFs. Third, ribosomes might reinitiate after translating the upstream ORF, an exercise that would seem to require 3′ to 5′ ribosome movement (backscanning) across the overlap region. Backscanning has been observed over a span of 80–90 nt (10), but an overlap of 92 nt was resistant to backscanning (9). To distinguish among the three possibilities with regard to VA-Tat RNA translation, we studied the effect of increasing the extent of the overlap of the two ORFs to more than 100 nt. If either of the first two expla-
nations pertains, increasing the overlap would not be expected to affect the translation of the Tat ORF. On the other hand, if the Tat ORF is translated by reinitiation after backscanning, an increase in the length of the overlap would be expected to decrease Tat ORF translation, since backscanning is observed only over short distances.

We exploited the six termination codons present in-frame with the upstream AUG of VA-Tat RNA (Fig. 2A, S1–S6) to extend the overlap. By site-directed mutagenesis, the in-frame stop codons were eliminated sequentially without altering the amino acids encoded by the Tat ORF. The resultant plasmids, pVA-Tat.S1, S1–3, and S1–5, contained mutations in the first stop codon, in the first three stop codons, and in the first five stop codons, respectively, yielding overlaps between the two ORFs of 10, 115, and 220 nt (Fig. 2A). The mutants were then tested in the HIV-CAT transactivation assay, giving results shown in Fig. 2B. Northern blot analysis indicated that similar levels of RNA were generated by the mutant and parental plasmids (data not shown). All constructs with overlapping ORFs produced decreased CAT activity compared with the wild type pVA-Tat construct, and extending the overlap from 10 to 115, or 220 nt, caused a decrease in translation of the downstream ORF. pVT.S1, which contains an overlap of 10 nt, was about 50% as active as wild type pVA-Tat, whereas pVT.S1–3 and pVT.S1–5, which have overlaps of 115 and 220 nt, respectively, both produced only about 20% of wild type activity. These results imply that Tat activity from RNA containing the S1 mutation only (10-nt overlap) may be partially accounted for

FIG. 3. The effect of overlapping ORFs in a pol II transcribed mRNA. A, schematic representation of pCMV-VT and its mutants. The start (I) and stop codons (S1–S6) of the upstream ORF are indicated. The downstream Tat ORF lies between the ATG and TAG. B, α-amanitin sensitivity of pCMV-VT transcription. Linearized pCMV-Tat, pCMV-VT, and pVA-Tat were transcribed in vitro in the presence or absence of 5 μg/ml α-amanitin, and the products were analyzed in a 7 M urea-8% polyacrylamide gel. Lane 1 contains the empty vector control. Positions and sizes of DNA markers are indicated (in nucleotides) on the right. C, Tat activity was measured in lysates of cells cotransfected with pHIV-LTR-CAT and pCMV-VT or its termination codon mutants (S1, S1–3, and S1–5). The plotted values are an average of four experiments normalized to 100 for CMV-Tat; the error bars indicate the standard deviation. D, Tat activity from cells cotransfected with pHIV-LTR-CAT and the I mutant of CMV.VT or its termination codon mutant derivatives (S1 and S1–3). Transactivation, a measure of Tat activity, is defined in Fig. 1. The plotted values are an average of two experiments normalized to 100 for CMV.VT, and the standard deviation is indicated as error bars.
by reinitiation requiring backscanning. Since increasing the overlap from 115 to 220 nt gave no further decrease in Tat activity, the Tat activity produced by these constructs may be due to leaky scanning. In other words, it seems that only 20% of the ribosomes scanning VA-Tat RNA failed to recognize the first AUG, and of those that do initiate translation at the upstream AUG, about 40% are able to backscan after translating the upstream ORF and reinitiate at the Tat ORF.

To test the effect of the overlaps on the translation of RNA generated by pol II, the same mutations were introduced into pCMV-VT which contained the CMV immediate early promoter, driving transcription of the VA-Tat sequence (Fig. 3A). The construct contains a polyadenylation signal downstream of the Tat sequence to ensure proper termination and processing of the pol II transcript, and the first 11 nt of VA-Tat sequence were deleted so as to remove the A box of the VA RNA gene promoter and impair pol III transcription. To verify that transcription of this plasmid was indeed dependent on pol II, its sensitivity toward α-amanitin was tested in a transcription assay in vitro (Fig. 3B). The plasmid was cut at +189 nt (with respect to the CMV transcription start site) with MfeI, and run-off transcription was performed in HeLa cell nuclear extract in the presence and absence of α-amanitin. Fig. 3B shows that transcription from pCMV-VT, like that from pCMV-Tat which also contains a pol II promoter (17), was eliminated by 5 μg/ml α-amanitin, whereas transcription from pVA-Tat containing pol III promoter was not. Thus transcription of pCMV-VT is dependent on the pol II promoter as a result of the deletion in its VA RNA gene promoter.

In the transactivation assay (Fig. 3C), overlap of the ORFs by 10 nt (S1) caused a decrease in translation of the downstream Tat ORF of CMV-Tat by about 40%, and increasing the length of the overlap (S1–3 and S1–5) caused a further decrease in expression. The additional decrease was small, however, possibly indicating that backscanning can occur over longer distances in the case of capped pol II transcripts than in the uncapped pol III transcripts. Mutation of the upstream AUG, which eliminates the upstream ORF, abolished the inhibition of Tat ORF translation in these stop codon mutants as expected (Fig. 3D), peaking that the effect of the stop codon mutations on Tat ORF translation is dependent on the integrity of the upstream ORF.

We also tested the effects of overlapping ORFs in a wheat germ cell-free translation system using both capped and uncapped mRNAs synthesized in vitro. As observed in vivo, the overlap of the two ORFs was inhibitory to translation of the downstream Tat ORF. In RNAs that contained long overlaps of the two ORFs, the translation product from the downstream ORF was reduced significantly (data not shown). Similar results were observed with both capped and uncapped RNA, confirming that capped RNA and uncapped RNA are translated by similar mechanisms that allow backscanning.

The 7-methyl guanosine cap structure that characterizes the 5’ end of eukaryotic mRNAs has a role in their stability, processing, and transport into the cytoplasm. It also enhances the efficiency with which mRNA binds initiation factor eIF4F (22). This factor being limiting in most eukaryotic cells, capped mRNA has a clear advantage for translation initiation (22–24). In this paper we assessed the significance of cap for the mechanism of translational initiation. We found that capped and uncapped mRNA are translated by similar mechanisms although with very different efficiencies.2 Both were scanned from the 5’ end of the RNA, indicating that the process of scanning does not require the cap. In addition, both allowed translation to initiate at a downstream ORF after the translation of an upstream ORF. When the two ORFs’ overlap, reinitiation appears to involve movement of the ribosomes in a backward direction along both capped and uncapped mRNA. We therefore concluded that the mRNA cap is not an essential feature of the translation initiation mechanism, although it is possibly required for efficient translation by virtue of recruiting eIF4F.

Eukaryotic cellular mRNA is believed to be synthesized exclusively by RNA polymerase II, but the mammalian cell is capable of utilizing an RNA pol III transcript as an mRNA to produce functional protein (15). Although pol III genes are transcribed with great efficiency, the translational efficiency of the resultant RNAs is very low due to the absence of the 5’ cap. Therefore it seems likely that one of the reasons why pol III genes have not been shown to code for proteins is the lack of the cap on their transcripts. Mechanisms such as trans-splicing and “cap-snatching” are used by trypanosomes (25) and influenza virus (26) to cap mRNAs that are transcribed by polymerases other than pol II (pol I and viral polymerase, respectively). It remains to be seen whether a comparable mechanism for capping of uncapped cellular RNA exists and allows the efficient translation of pol III transcripts.

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