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Functional mRNA Can Be Generated by RNA Polymerase III

SHOBHA GUNNERY AND MICHAEL B. MATHEWS*
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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Eukaryotic cellular mRNA is believed to be synthesized exclusively by RNA polymerase II (pol II), whereas pol I produces long rRNAs and pol III produces 5S rRNA, tRNA, and other small RNAs. To determine whether this functional differentiation is obligatory, we examined the translational potential of an artificial pol III transcript. The coding region of the human immunodeficiency virus type 1 *tat* gene was placed under the control of a strong pol III promoter from the adenovirus type 2 VA RNA_I gene. The resultant chimera, pVA-Tat, was transcribed accurately in vivo and in vitro and gave rise to Tat protein, which transactivated a human immunodeficiency virus-driven chloramphenicol acetyltransferase reporter construct in transfected HeLa cells. pol III-specific mutations down-regulated VA-Tat RNA production in vivo and in vitro and dramatically reduced chloramphenicol acetyltransferase transactivation. As expected for a pol III transcript, VA-Tat RNA was not detectably capped at its 5′ end or polyadenylated at its 3′ end, but, like mRNA, it was associated with polysomes in a salt-stable manner. Mutational analysis of a short open reading frame upstream of the Tat-coding sequence implicates scanning in the initiation of VA-Tat RNA translation despite the absence of a cap. In comparison with *tat* mRNA generated by pol II, VA-Tat RNA was present on smaller polysomes and was apparently translated less efficiently, which is consistent with a relatively low initiation rate. Evidently, human cells are capable of utilizing pol III transcripts as functional mRNAs, and neither a cap nor a poly(A) tail is essential for translation, although they may be stimulatory. These findings raise the possibility that some cellular mRNAs are made by pol I or pol III.

Eukaryotic RNA is synthesized by three distinct types of cellular RNA polymerases, polymerase I (pol I), pol II, and pol III. Although the three polymerases employ some common transcription factors, they are recruited by distinct promoter elements to different classes of genes. This results in a marked degree of specialization such that individual classes of RNA are produced by each polymerase: the large rRNAs are produced by pol I; mRNA and certain small RNAs are produced by pol II; and tRNA, 5S rRNA, and certain other small RNAs are produced by pol III. It seems that this discrimination is respected absolutely and in all organisms; for example, no cellular mRNA is known to be produced by any polymerase other than pol II. This specialization with respect to RNA products could reflect underlying characteristics of the polymerases that restrict the kinds of transcripts produced: the intranuclear compartmentalization of pol I within the nucleolus might be one example, and the tendency of pol III to terminate transcription in runs of thymidine residues might provide another. Alternatively, a functional limitation could be imposed by some property of the transcript; for example, its fate could be determined by a 5′-terminal cap or 3′-terminal poly(A) tail. On the other hand, it is also possible that the specialization, despite its universality, merely perpetuates some archaic property of eukaryotes without any necessary purpose in contemporary organisms.

Few attempts to resolve this issue experimentally have been made, and the results to date have been inconclusive. Some studies have examined the competence of pol I transcripts to support protein synthesis. Fleischer and Grummt (14) reported that simian virus 40 T antigen and bacterial chloramphenicol acetyltransferase (CAT) can be expressed from a pol I promoter in microinjected or transfected cells, at both transcriptional and translational levels. The expression was α-amanitin insensitive and unaffected by pol II enhancer elements, arguing against the possibility that transcription actually arose from a cryptic pol II promoter. On the other hand, Lopata et al. (40), who used a similar pol I-CAT chimera, described the production of aberrantly initiated transcripts which were polysome associated and translated. The authentic transcript initiating at the pol I initiation site was not polysome associated and was unlikely to be translated. Whereas synthesis of the authentic transcript was unresponsive to pol II enhancer elements, the production of the aberrantly initiated transcripts and CAT activity were stimulated by the pol II enhancer. These findings called into question the earlier conclusion that pol I transcripts can support protein synthesis in vivo. Further doubts were added by the observation that herpesvirus thymidine kinase RNA generated by pol I transcription was unstable, nonpolyadenylated, and largely nucleolar (65). Similar controversy surrounds mRNA synthesis by pol III. It has been reported that β-globin transcripts generated by pol III were spliced and polyadenylated (8) and that polyadenylated thymidine kinase RNA was produced and transported efficiently into the cytoplasm from a chimeric gene under the control of a pol III promoter (38), but these conclusions have also been disputed (64).

One of the unique features of eukaryotic mRNA is the presence of a characteristic 7-methyl guanosine (m^7G) cap at its 5′ end, which is added during transcription, shortly after elongation has commenced (30, 55, 60). Functions have been attributed to the cap at numerous stages of gene expression: in RNA stabilization, splicing, transport, and translation. The scanning model of translational initiation proposes that ribosomal subunits bind to the mRNA 5′ end and move along the strand, in processes that are facilitated by the cap, until they encounter the first translational initiation codon in a favorable sequence context, whereupon translation is initiated (32). The first-AUG rule holds for many natural and engineered mRNAs and the presence of an upstream AUG or open reading frame

* Corresponding author. Mailing address: Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724. Phone: (516) 367-8374. Fax: (516) 367-8815.

(ORF) has deleterious effects on the translation of downstream ORFs, as predicted by the scanning hypothesis. Some viral and cellular mRNAs do not follow this rule, however (66). Their translation is not 5′ end dependent, nor does it initiate at the first AUG; instead, the ribosomes gain access to the ORF through an internal ribosome entry site (IRES). A stimulatory role in translational initiation has also been assigned to the 3′ poly(A) tract (for a review, see reference 29), although the existence of translationally active nonpolyadenylated mRNAs argues that this modification is not indispensable.

In this study we examined the translational potential of a pol III transcript to determine whether there is an obligatory link between pol II transcription and mRNA function. The coding region of the human immunodeficiency virus type 1 (HIV-1) *tat* gene was placed under the control of a pol III promoter derived from the adenovirus type 2 VA RNA_I gene. The Tat-coding sequence is short and lacks runs of thymidines which constitute the termination signal for pol III transcription (5), and the biological activity of the Tat protein can be assayed in vivo by cotransfection with a reporter construct containing the HIV-1 promoter which is transactivated by Tat. We show that the chimeric plasmid pVA-Tat, thus constructed, was transcribed in vivo and in vitro to generate the predicted RNA, which was neither capped nor polyadenylated. Its transcription was dependent on pol III-specific signals and was insensitive to low concentrations of α-amanitin, as expected of a pol III transcript. Like normal cellular mRNA, VA-Tat RNA, although uncapped and nonpolyadenylated, was associated with polysomes in a salt-stable manner. When cotransfected with pHIV-CAT, pVA-Tat elicited a large increase in CAT activity, showing that it generates functional mRNA which is translated. We conclude that active Tat can be generated by translation of an RNA produced by pol III. Although Tat expression was weak in comparison with expression from a construct driven by a strong pol II promoter (pCMV-Tat), these findings imply that pol III transcripts can be transported properly within the cell and assembled with translational components. The relatively inefficient translation of VA-Tat RNA seems to be due at least in part to an impediment to initiation, suggesting that pol II transcripts may predominate in the mRNA population because other transcripts are handicapped at various stages in gene expression.

MATERIALS AND METHODS

Plasmid construction. pVA-Tat contains the first coding exon sequence of the HIV-1 *tat* gene flanked by the promoter and termination sequences of the adenovirus type 2 VA RNA_I gene. It was constructed as follows: the *Xba*I-to-*Sal*I fragment from pMHIs6 (45), containing the VA RNA sequence, was ligated into the equivalent sites in the polylinker sequence of the pUC119 to yield pVA-ls6. The *Bam*HI site in the polylinker in pVA-ls6 was deleted by cutting the plasmid with *Kpn*I and *Xba*I, blunting the ends with the Klenow fragment of DNA pol I, and ligating the ends, resulting in pVA-ls6-B. Then, the *Hin*dIII-to-*Afl*III fragment (274 bp) containing the *tat* sequence from pCMV-Tat was ligated between the *Bam*HI and *Bgl*II sites of pVA-ls6-B. Before ligation, all sites were blunted by filling in the 3′ recess with the Klenow fragment of DNA pol I. Mutations were made by the site-directed mutagenesis procedure of Zoller and Smith (73). Mutations at sites A and B are described below. Mutations at sites I and S changed the translation start codon, AUG (nucleotides [nt] +44 to +46), and the termination codon, TAG (between nt +86 and +88), of the upstream ORF to GCG and CAG, respectively. Mutant I+S is a double mutant with both the initiator and termination codons mutated. Runs of thymidines were created at +100 (mutant T1), upstream of the Tat-coding region, and at +234 (mutant T2), in the coding region, without altering the amino acid sequence of the protein. All mutant constructs were sequenced to confirm the mutation sites. pGEM-Tat120 was constructed by inserting the *Hin*dIII-to-*Mun*I fragment from pCMV-Tat, containing the 5′ end of the *tat* sequence, between the *Hin*dIII and *Eco*RI sites of pGEM-1 (Promega). Plasmid pT7 VA-Tat was transcribed in the presence or absence of m^7GpppG to synthesize capped and uncapped VA-Tat RNA in vitro (22a).

Other plasmids. pHIV-CAT is a deletion construct of pU3RIII (p-167 [58]) that contains the CAT reporter gene driven by −167 to +82 of the HIV-1 long terminal repeat (LTR). pΔS-CAT contains a 4-nt deletion from +35 to +38 in the TAR region of pHIV-CAT which makes it nonresponsive to Tat transactivation. pCMV-Tat is the same as pBC/CMV/t2 (10) and contains the immediate-early gene promoter of human cytomegalovirus (CMV) upstream of the *Sal*I-to-*Kpn*I fragment of the *tat* gene, encompassing the first coding exon and 304 bp of the adjacent intron sequence. pαa+c contains the human α-globin gene under the control of its own promoter (69). pβ-Gal expresses β-galactosidase (β-Gal) under the control of the CMV immediate-early promoter (pON260 [67]).

HIV LTR transactivation assay. HeLa cells grown to 50% confluency in 6-cm-diameter plates were transfected by the calcium phosphate method (25). pUC119 was added as a carrier to a total of 15 μg per plate. All plates were transfected with 1 μg of pβ-Gal. The cells were harvested at 48 h posttransfection, and lysate prepared by freezing and thawing was assayed for CAT enzyme activity by standard procedures (25). The β-Gal assay was performed as described by Herbomel et al. (23). β-Gal activity was expressed as the optical density at 420 nm obtained with 25 μl of the lysate incubated at 37°C for 1 h; β-Gal activity was used to normalize the CAT enzyme activity for transfection efficiency. The relative CAT activity is defined as the percentage of chloramphenicol acetylated by 1 μl of the cell lysate at 37°C in 1 h, corrected for β-Gal activity.

RNA analysis. Cytoplasmic RNA was prepared following lysis with Nonidet P-40 as described by Anderson et al. (1). Total cytoplasmic RNA was used for Northern (RNA) blot analysis with an RNA probe complementary to the 5′ end of the VA RNA sequence (26). RNase protection analysis was conducted as described by Laspia et al. (37). An RNA probe complementary to the *tat* sequence was synthesized with T7 RNA polymerase from pGEM-Tat120 linearized with *Nhe*I. An RNA probe complementary to the α-globin sequence was made with SP6 polymerase from pαa+c linearized with *Bam*HI by using [α-^{32}P]UTP from ICN Radiochemicals, Inc. Oligo(dT) cellulose column chromatography of RNA was performed by a standard protocol (41). RNA was 3′ end labeled with [5′-^{32}P]pCp and T4 RNA ligase as described by England and Uhlenbeck (13) and 5′ end labeled with [α-^{32}P]ATP and polynucleotide kinase as described by Mellits and Mathews (45).

Polysome isolation. Cells from 10-cm-diameter plates were harvested at 48 h posttransfection and suspended in 0.2 ml of TSM buffer (10 mM Tris-Cl [pH 7.6], 150 mM NaCl, 2 mM $MgCl_2$) containing 0.5% Nonidet P-40. After 15 min on ice, cell debris was removed by centrifugation, and 100 μl of the supernatant was layered on 3.5 ml of 15% sucrose in TSM. The concentration of NaCl was adjusted to 0.5 M in the remaining 100 μl of the lysate, which was then layered onto 15% sucrose in TSM containing 0.5 M NaCl. After centrifugation in a Beckman TLA 100.3 rotor at 75,000 rpm for 45 min, pellets were suspended in TSM And RNA was isolated for analysis. To examine polysome profiles, cell lysates were prepared as described above, layered on an 11-ml 15 to 35% sucrose gradient, and centrifuged at 35,000 rpm for 2 h in a Beckman SW 60.1 rotor. The gradients were pumped through an ISCO UV monitor, and 1-ml fractions were collected for RNA extraction.

Capping reaction. RNA was 5′ end labeled with vaccinia virus guanylyltransferase (47). Reactions were performed in a final volume of 50 μl containing 25 mM Tris-Cl (pH 7.8), 2 mM $MgCl_2$, 1 mM dithiothreitol, 60 mM NaCl, 6 mM KCl, 80 U of RNasin, 0.1 mM *S*-adenosylmethionine, 5 μM GTP, 0.1 mCi of [α-^{32}P]GTP, 5 μg of RNA, and 4 U of guanylyltransferase (Gibco). The reaction mixture was incubated at 37°C for 30 min, after which 10 mM EDTA and 0.4% sodium dodecyl sulfate were added to terminate the reaction. The RNA was extracted with phenol and chloroform, ethanol precipitated, and then subjected to hybrid selection for the VA RNA sequences with pVA or for *tat* sequences with pCMV-Tat as described by Ausubel et al. (2).

Immunoprecipitation. Polyclonal antibody directed against m^7G cap (kindly provided by Ted Munns and E. Lund) was bound to protein A-Sepharose CL-4B beads and used for immunoprecipitating RNA as described by Terns et al. (70).

RNase H analysis of RNA. Reaction mixtures containing 5 μg of end-labeled RNA were incubated with RNase H in the presence or absence of 0.3 μg of poly(dT) as described by Mercer and Wake (46). The products of the reactions were analyzed by electrophoresis in an 8% polyacrylamide–7 M urea gel, either directly or after hybrid selection with pVA-Tat.

RESULTS

Expression of pVA-Tat. Cellular mRNA and primary transcripts made by pol II appear to follow a defined pathway through the nucleus and into the cytoplasm as they are processed, transported, and utilized for translation (for a review, see reference 68). Although abundant and stable cytoplasmically located transcripts are produced by pol III, none of these is known to encode a protein. To determine whether transcription by pol III is compatible with recruitment of the RNA product for translation, we constructed a vector, pVA-Tat, in which the HIV-1 Tat-coding sequence is driven by the adenovirus VA RNA promoter, a strong pol III promoter. Its chief

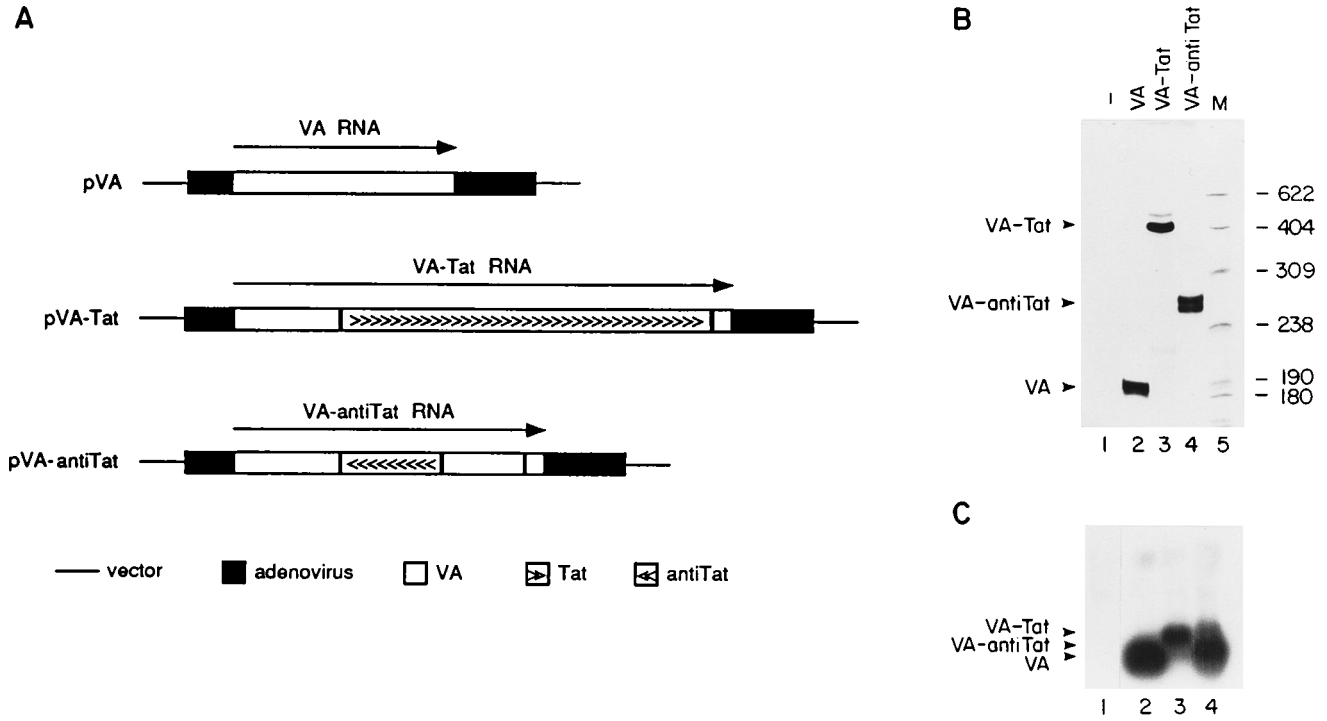


FIG. 1. pol III transcripts. (A) Schematic diagram of the plasmids. (B) Expression of the plasmids in vitro with a pol III transcription system (44). The RNA products were analyzed in an 8% polyacrylamide–7 M urea gel. End-labeled DNA markers (pBR332 cut with *Hpa*II) are shown on the right (lane M; sizes in nucleotides). (C) Expression of the plasmids in vivo. RNA from cells transfected with the respective plasmids was analyzed by Northern blot analysis with the 5′ VA probe.

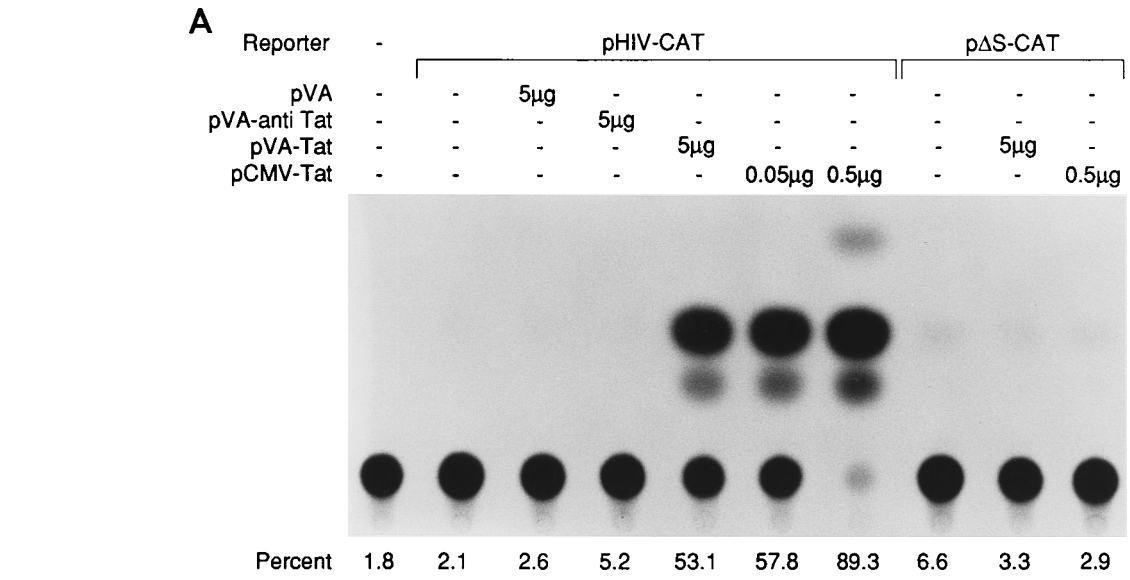
elements are intragenic in that two essential regions of the VA RNA promoter known as the A and B boxes are present downstream of the transcriptional start site (15, 21; for a review, see reference 19). Therefore, the promoter sequence is transcribed as the 5′ end of the RNA. This promoter has been used previously by us and others to generate short RNAs in vivo (22, 38). Inserted between the internal promoter region of VA RNA and its termination signal, which lies in a run of four thymidine residues (Fig. 1A), is the first coding exon sequence of the HIV-1 Tat protein. The substitution removes VA RNA sequences that are essential for its role in translational control (43).

Transcription by pol III was expected to give rise to an RNA of 368 nt, consisting of 75 nt from the VA RNA promoter followed by 277 nt of the *tat* sequence and 16 nt of the VA RNA sequence and ending in the 3′ run of four uridines. Both in a pol III transcription system (Fig. 1B) and in transfected cells (Fig. 1C), pVA-Tat produced the predicted RNA. The parent plasmid pVA, encoding VA RNA_I, and a control plasmid (pVA-antiTat, containing a portion of the *tat* gene inserted in the opposite orientation) also produced the transcripts expected (Fig. 1).

To examine the translational potential of the VA-Tat transcript, we assayed its ability to produce Tat protein in transfected cells, taking advantage of Tat's capacity to transactivate expression from the HIV promoter containing the TAR region. HeLa cells were transfected with the reporter plasmid pHIV-CAT, and CAT enzyme activity in cell lysates was subsequently measured. Cotransfection with pVA-Tat caused a large increase in CAT activity (Fig. 2A), whereas plasmids that do not encode Tat-coding sequence (pVA and pVA-antiTat) elicited no effect (Fig. 2A). Comparable amounts of RNA accumulated in cells transfected with all three plasmids, as determined by Northern blot analysis (Fig. 1C). The response to pVA-Tat was concentration dependent (Fig. 2B), but it saturated at considerably lower levels than did the response to pCMV-Tat, which contains the Tat-coding sequence under the control of the immediate-early CMV promoter (Fig. 2A).

Transactivation of the HIV LTR by Tat is dependent on the protein's interaction with TAR RNA and is accordingly sensitive to mutations in the TAR region (for a review, see reference 51). To verify the specificity of transactivation by pVA-Tat, a mutant reporter construct, pΔS-CAT, containing a 4-nt deletion within the TAR region, was cotransfected with pVA-Tat or with pCMV-Tat. No CAT activity was observed (Fig. 2A), confirming that transactivation by pVA-Tat is due to the production of Tat protein.

VA-Tat expression is dependent on pol III transcription. The data in Fig. 2 demonstrate the synthesis of functional Tat protein from pVA-Tat and lead to the presumption that Tat can be made from a pol III transcript. However, they do not exclude the possibility of a cryptic pol II promoter directing the synthesis of VA-Tat RNA. Both in vivo and in vitro, synthesis of VA-Tat RNA exhibited the sensitivity to the drug α-amanitin that would be expected of a pol III transcript (data not shown). For a more definitive test, we examined the effects of mutations that are deleterious to pol III transcription and would not be expected to affect transcription by pol II. Three mutations targeted the two essential elements of pol III transcription within the VA RNA gene promoter, the A and B boxes, situated between nt 14 and 24 and nt 59 and 69, respectively (Fig. 3A). Mutational analysis of these two regions of the VA RNA promoter has shown that substitutions of G16 (the guanosine nucleotide at +16) and G60 are very deleterious to transcription (56). Accordingly, U14 and G16 were mutated to adenosine residues by oligonucleotide-directed mutagenesis to



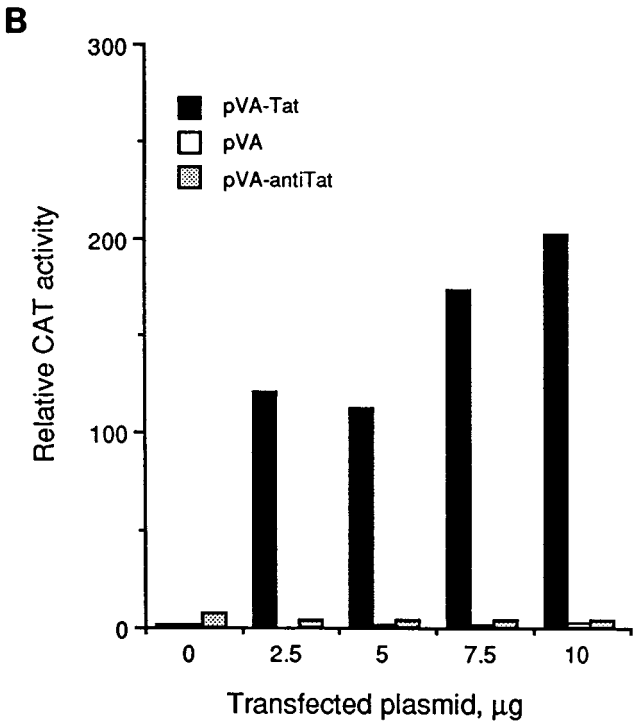
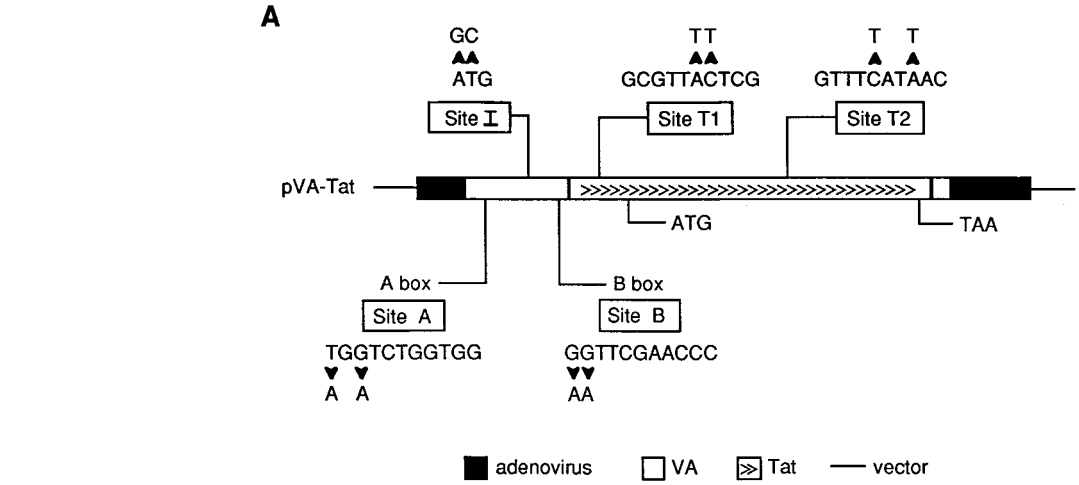


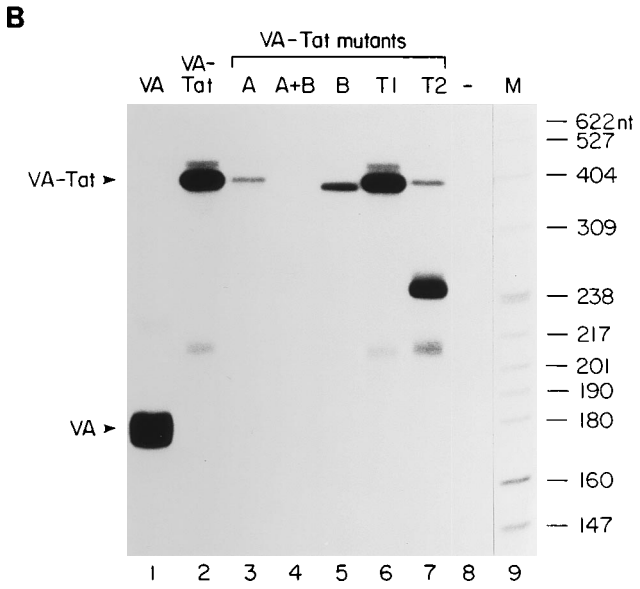
FIG. 2. Functionally active Tat protein is produced in pVA-Tat-transfected cells. (A) HIV LTR transactivation assay. HeLa cells were transfected with pHIV-CAT or the deletion mutant plasmid pΔS-CAT alone or with specified amounts of pVA, pVA-antiTat, pVA-Tat, or pCMV-Tat. The cell lysates were assayed for CAT enzyme activity. The actual CAT enzyme activity is indicated as the percentage of chloramphenicol acetylated by 25 μl of the cell extract in 1 h. (B) Dose-response curve of pVA-Tat, pVA, and pVA-antiTat transactivation activities. The relative CAT enzyme activity is shown as CAT units per microliter of the lysate, corrected for β-Gal activity (an indicator of transfection efficiency).

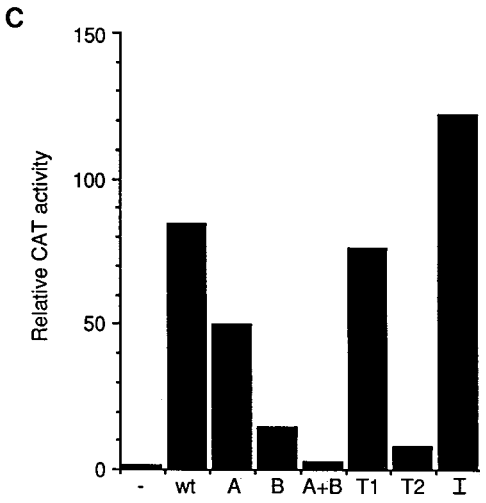
create the site A mutant, and G59 and G60 were changed to adenosine residues to create the site B mutant (Fig. 3A). A double mutant (A+B) was also constructed where both elements were mutated simultaneously. As expected, these mutations greatly decreased transcription in vitro in a pol III transcription system (Fig. 3B, lanes 3 and 5). Combining the A and B box mutations essentially abolished transcription (lane 6).

Two further mutants were made by creating pol III termination signals within the VA-Tat sequence. Without altering the amino acid-coding sequence of the gene, a run of five thymidine residues was placed before the protein-coding sequence (site T1 [Fig. 3A]) and a second T-rich stretch (TTTTATT) was inserted within the Tat-coding region (site T2 [Fig. 3A]). Both of these mutations were expected to produce truncated RNA; the T1 mutant RNA should lack the entire protein-coding sequence, while the T2 mutant RNA should encode a protein lacking the C-terminal 33 amino acids of the Tat protein. The C-terminal sequence of Tat includes the basic domain, which is essential for its nuclear localization and interaction with TAR RNA, so this mutation should eliminate the transactivation function. In the pol III transcription system, the termination signal at site T2 caused truncation of most of the RNA produced (Fig. 3B, lane 7). Surprisingly, the mutation at site T1 had no effect (Fig. 3B, lane 6). The reason for this is not clear, because the mutation creates a run of five T residues situated in a GC-rich context which, according to earlier work (5), should make a strong signal for pol III termination.

The ability of these VA-Tat mutants to generate functional Tat was tested in the transactivation assay. Figure 3C shows that the promoter mutations A and B separately reduced CAT enzyme accumulation; together in one mutant (mutant A+B), they almost eliminated transactivation. The effective termination signal, T2, severely reduced CAT activity, while the ineffective signal, T1, was nearly as active as the wild-type pVA-Tat in the CAT transactivation assay. Examination of RNA from the transfected cells by Northern blot analysis (Fig. 3D) confirmed that T2 RNA was stable, although truncated, whereas T1 RNA was similar to wild-type VA-Tat RNA in size and amount, as expected from Fig. 3B. When installed in a pol II transcription unit, neither mutation T1 nor T2 had any effect on transactivation by Tat, as expected (data not shown). Little RNA was detected from mutants A and B, and even less was detected from the double mutant, A + B. These results are consistent with the in vitro transcription data (Fig. 3B) and with the Tat transactivation activities of the mutants (Fig. 3C), with one exception. Although transactivation was reduced by







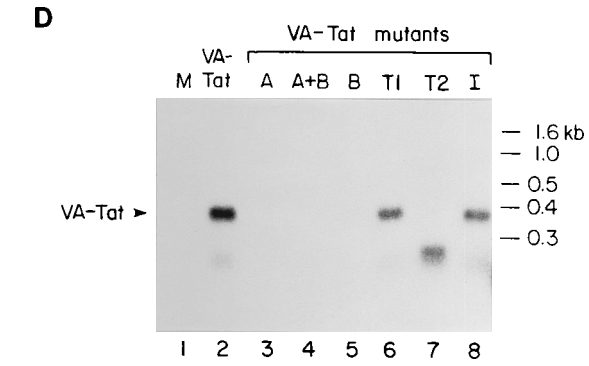


FIG. 3. Effect of mutations in RNA pol III transcription signals on transactivation by pVA-Tat. (A) Sites of the mutations in pVA-Tat. (B) Products of in vitro transcription by pol III of pVA (lane 1), wild-type pVA-Tat (lane 2), and mutant pVA-Tat constructs (lanes 3 to 7) analyzed in an 8% polyacrylamide–7 M urea gel. Lane 8 contains products of a control reaction carried out in the absence of template DNA. End-labeled DNA markers are shown on the right (lane M). (C) HIV CAT transactivation by wild-type (wt) and mutant pVA-Tat. The relative CAT enzyme activity is shown as CAT units per microliter of the lysate, corrected for β-Gal activity (an indicator of transfection efficiency). The bar labeled with the minus sign indicates basal activity from pHIV-CAT alone. (D) Total cytoplasmic RNA from cells transfected with wild-type (lane 2) or mutant (lanes 2 to 8) pVA-Tat was subjected to Northern blot analysis with the 5′ VA probe. The position of wild-type VA-Tat RNA is indicated on the left, and RNA markers are shown on the right.

the A mutation by only about 40% (Fig. 3C), very little RNA was evident in the transfected cells, which is suggestive of an RNA detection problem. As expected, the A mutation reduced expression in the A+B mutant, relative to expression from the B mutant. This anomaly notwithstanding, the data from the mutants indicate that pol III transcription is essential for the production of VA-Tat RNA and for transactivation of HIV CAT. Therefore, the VA-Tat RNA that is translated is indeed a pol III product and not derived from a cryptic pol II promoter.

VA-Tat RNA is not capped or polyadenylated. Unlike pol II transcripts, most pol III RNAs are neither polyadenylated at the 3′ end nor capped at the 5′ end, so we would expect VA-Tat transcripts to lack these posttranscriptional modifications. To investigate the polyadenylation status of VA-Tat RNA, RNA from pVA-Tat-transfected cells was subjected to oligo(dT) cellulose column chromatography. The column fractions were examined by Northern blot analysis (Fig. 4A, top). VA-Tat RNA did not bind to the column and was recovered with poly(A)$^-$ species in the flowthrough and wash fractions (Fig. 4A, lanes 3 to 5). As a control, the cells were cotransfected with plasmid αa+c, which expresses the α-globin gene under the direction of pol II. The α-globin mRNA bound to the oligo(dT) cellulose column (Fig. 4A, bottom) and was

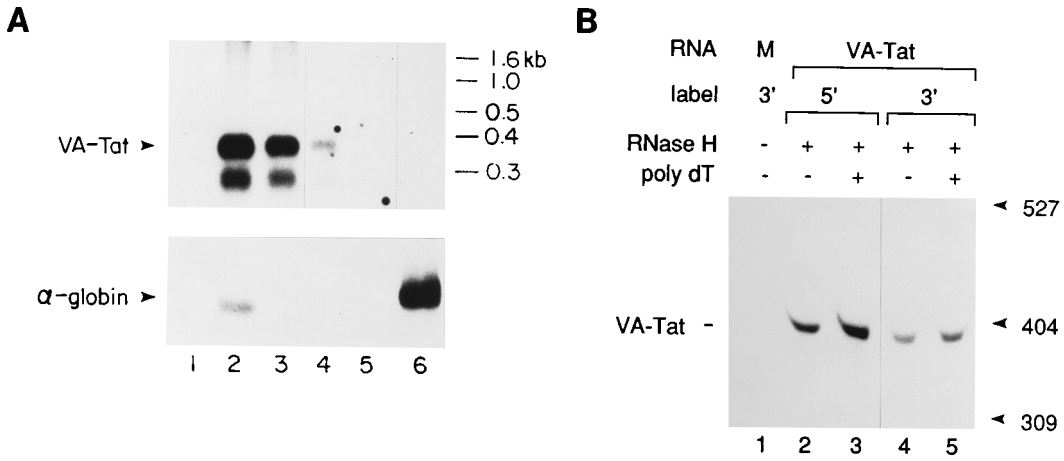


FIG. 4. VA-Tat RNA is not polyadenylated. (A) RNA from cells transfected with pVA-Tat and pαa+c was fractionated in an oligo(dT) cellulose column. The starting material (lane 2), first flowthrough fraction (lane 3), 0.1 M NaCl wash fractions (lanes 4 and 5), and eluate (lane 6) were subjected to Northern blot analysis with the 5′ VA probe (top panel). The bottom panel shows RNase protection analysis with a probe complementary to α-globin. RNA from mock-transfected cells was run in lane 1. Lanes 1 to 5 contain equivalent portions of RNA; lane 6 contains three times this amount. RNA marker sizes are given on the right. (B) RNA from cells transfected with pVA-Tat (lanes 2 to 5) or mock-transfected cells (lane 1) was 3′ or 5′ end labeled, selected by hybridization with pVA-Tat DNA, and then subjected to RNase H digestion in the presence or absence of poly(dT), as indicated. Reaction products were analyzed in an 8% polyacrylamide–7 M urea gel. End-labeled DNA markers in nucleotides are given on the right.

eluted with 0.5 M salt as expected for a polyadenylated RNA. Because this assay might not detect RNAs with poly(A) tails of less than 20 nt (46), we conducted a more sensitive test with RNase H, an enzyme that degrades the RNA strands of an RNA:DNA heteroduplex. The RNA from pVA-Tat-transfected cells was labeled at its 5′ end with polynucleotide kinase and [γ-^{32}P]ATP or at its 3′ end with RNA ligase and [^{32}P]pCp. VA-Tat RNA was isolated by hybrid selection and incubated with RNase H in the presence or absence of poly(dT). Under these conditions, synthetic poly(A) was completely digested provided poly(dT) was present (data not shown). Regardless of the site of the end label, VA-Tat RNA was unaffected by RNase H whether poly(dT) was present or not (Fig. 4B, lanes 2 to 5), which is consistent with its poly(A)$^-$ status. Poly(A) tails as short as 5 nt have been detected in this way (11), thereby placing an upper limit of five on the number of A residues that might be present at the 3′ end of VA-Tat RNA. We also used a label transfer protocol to determine the 3′-terminal nucleotide of VA-Tat RNA. After being labeled with [^{32}P]pCp at its 3′ end, VA-Tat RNA was digested with alkali, and the resultant 3′ nucleoside monophosphates were separated by paper electrophoresis (data not shown). Most of the label (about 70%) was in UMP, as expected if the VA-Tat RNA terminated at the VA RNA termination site. Some label (about 25%) was also detected in AMP, raising the possibility that a fraction of the molecules end in adenosine or carry a short oligo(A) tract. Such tracts, if they existed, would be released by digestion of end-labeled VA-Tat RNA with a mixture of RNases T_1 and A, which cleave after U, C, and G residues. Upon gel electrophoresis, the vast majority of the label migrated as a trinucleotide or faster (mostly as a mononucleotide), suggesting that the A label is likely due to the addition of pCp to internal positions of broken VA-Tat RNA molecules. We therefore conclude that little or none of the VA-Tat RNA carries a poly(A) tail.

Primer extension analysis precisely mapped the 5′ end of VA-Tat RNA to the start site of VA RNA (data not shown), and the fact that VA-Tat RNA can be 5′ end labeled by polynucleotide kinase (Fig. 4B, lanes 2 and 3) implies that some or all of it has a free 5′ end, at least after phosphatase treatment. These data suggested that the 5′ end of VA-Tat RNA is probably uncapped and that it might be a substrate for the capping reaction. Guanylyltransferase, better known as capping enzyme, transfers a guanylyl nucleotide onto the 5′ end of an RNA provided the RNA is not already capped and carries a 5′ di- or triphosphate nucleotide, therefore excluding 5′ ends created by degradation (47). Consequently, guanylyltransferase, in the presence of [α-^{32}P]GTP, should label VA-Tat RNA if it is produced by pol III. RNA from transfected cells was subjected to the capping reaction in the presence of labeled GTP, followed by hybrid selection for VA RNA or *tat* RNA sequences and analysis by gel electrophoresis. The VA-Tat RNA gave a band of the expected size (Fig. 5A, lanes 2 and 7), indicating that it is uncapped in vivo. As a positive control, a known pol III product, VA RNA from cells transfected with pVA, was also labeled in the capping reaction (Fig. 5A, lane 3). On the other hand, RNA from cells transfected with pCMV-Tat remained unlabeled, as expected for a pol II transcript which is already capped in vivo. To confirm this result, we used antibodies directed against the 5′ cap structure to examine VA-Tat RNA for the presence of caps. The antibody precipitated capped VA-Tat RNA, synthesized in vitro by T7 RNA polymerase in the presence of m^{7}GpppG (Fig. 5B, lanes 4 to 6), but not uncapped VA-Tat RNA, synthesized in the absence of the methylated nucleotide (Fig. 5B, lanes 1 to 3). When cytoplasmic RNA isolated from transfected cells was analyzed with the antibody, no VA-Tat RNA was detected in the immunoprecipitate, suggesting that little or none of it was capped (Fig. 5B, lanes 7 to 9). Thus, the VA-Tat RNA produced in transfected cells is not detectably polyadenylated or capped, which is consistent with transcription by pol III.

Polysomal association of VA-Tat RNA. The demonstration that VA-Tat RNA can be capped shows that some, though not necessarily all, of this RNA lacks a cap structure. Capped RNA is translated better than uncapped RNA in vitro (62) and in vivo (17, 57), so it was conceivable that an undetected, small fraction of the VA-Tat RNA is capped and that this RNA is translated whereas the uncapped VA-Tat RNA is not recruited by the ribosomes for translation. To test this hypothesis, we analyzed the RNA that is associated with polysomes from

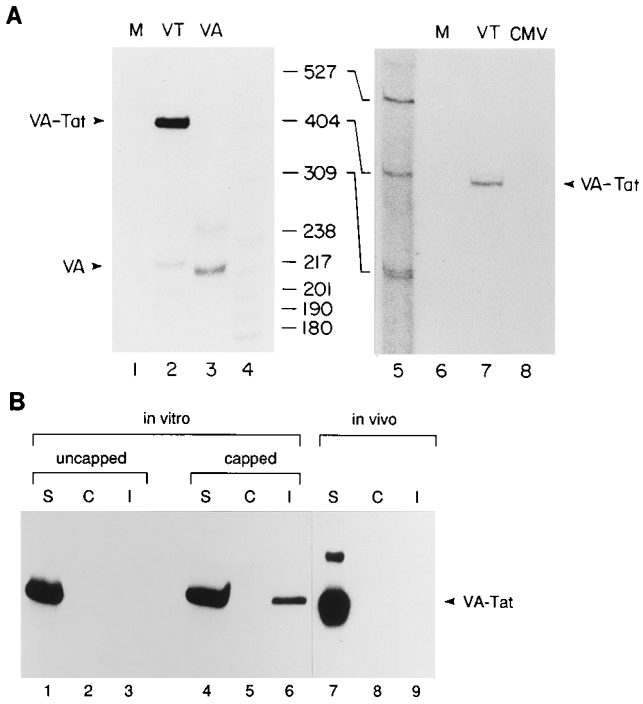


FIG. 5. VA-Tat RNA is uncapped. (A) The RNA from cells transfected with pVA-Tat (VT; lanes 2 and 7), pVA (VA; lane 3), or pCMV-Tat (CMV; lane 8) or from mock-transfected cells (M; lanes 1 and 6) was subjected to the capping reaction in the presence of [α-^{32}P]GTP followed by hybrid selection for the VA RNA sequence (lanes 1 to 3) or the *tat* sequence (lanes 6 to 8). The selected RNA was analyzed in an 8% polyacrylamide–7 M urea gel. Lanes 1 to 5 contain equivalent portions of the RNA; lane 6 contains three times this amount. End-labeled DNA markers (lanes 4 and 5) are given between the panels (in nucleotides). (B) Uncapped (lanes 1 to 3) or capped (lanes 4 to 6) VA-Tat RNA transcribed in vitro or RNA from pVA-Tat-transfected cells (lanes 7 to 9) was immunoprecipitated with a cap-specific antibody and analyzed in an 8% polyacrylamide–7 M urea gel or by Northern blot analysis, respectively. Lanes marked "S" contain the starting material; lanes marked "C" and "I" contain material immunoprecipitated in the absence or presence of the anticap antibody, respectively.

transfected cells. RNAs that are actively translated are tightly bound to polysomes and cannot be dislodged by high-salt treatment, unlike many of the accessory components of the translational apparatus (including the protein kinase DAI and VA RNA, which associates with it). Therefore, if uncapped VA-Tat RNA is translated in the transfected cells, it should be associated with the polysomal fraction of the cell in a salt-stable manner.

Polysomal pellets were prepared from transfected cell lysates under low-salt or high-salt conditions, and the RNA extracted from the pellets was examined by Northern blot analysis with a probe complementary to the 5′ end of VA RNA. VA-Tat RNA was present in the polysomal pellet prepared under both low-salt and high-salt conditions (Fig. 6A, lanes 5 and 11). VA RNA, in contrast, was found in the polysomal pellet prepared under the low-salt conditions but not under the high-salt conditions (Fig. 6A; compare lanes 6 and 12). Like VA-Tat RNA, CMV-Tat RNA was also associated with the polysomal pellet in a salt-stable manner, as would be expected of an mRNA (data not shown). To determine whether the VA-Tat RNA that is stably associated with the polysomal pellet was also uncapped, we subjected it to the guanylyltransferase reaction followed by hybrid selection for VA RNA sequences as described above. The ribosome-associated RNA was labeled in the capping reaction (Fig. 6B, lane 4), demonstrating that uncapped VA-Tat RNA is recruited for translation by the ribosomes.

Similar results were obtained when polysomes were examined by sucrose gradient sedimentation in the presence of a high concentration of salt. Figure 6C displays the distribution of *tat* RNA from cells transfected with pVA-Tat or pCMV-Tat. VA-Tat RNA was found preferentially in the monosome peak with small amounts in the disome peak and traces in the polysome region of the gradient. CMV-Tat, by contrast, was present in the monosome peak, with larger amounts in the disome peak and substantial amounts in the polysome region. Thus, both species are loaded onto ribosomes but CMV-Tat RNA is more efficiently incorporated into large polysomes than is VA-Tat RNA. A shift to smaller polysomes often accompanies a decrease in the rate of initiation on an mRNA species. Given the small size of *tat* mRNA, it is difficult to draw firm conclusions in this case, but the differential loading suggests that initiation may be the rate-limiting step for VA-Tat RNA translation.

Finally, in case there exists a small fraction of the VA-Tat RNA which is capped and preferentially associated with translating ribosomes, we examined fractions from the gradient by immunoprecipitation with anticap antibody (Fig. 6D). Again, no capped VA-Tat RNA was detected in any fraction, confirming our previous conclusion on this point.

Do ribosomes scan VA-Tat RNA? It appears from the data of Fig. 2B that Tat production from pVA-Tat was about 100-fold less efficient than Tat production from pCMV-Tat, and Fig. 6C shows that VA-Tat RNA is less efficiently loaded onto polysomes than is CMV-Tat RNA. Several factors may contribute to the low translational efficiency. One of these is the existence of an AUG initiator codon in the VA RNA sequences upstream of the Tat-coding region. The 5′ untranslated region of VA-Tat RNA is 123 nt long and contains an ORF between nt 44 and 88 that is out of frame with that of Tat. Such upstream ORFs can reduce the translation of a downstream ORF, especially if the ORF begins with an AUG in a favorable context, an observation that is commonly interpreted to imply that initiating ribosomal subunits first bind to the 5′ cap and then scan along the mRNA until they encounter a suitable AUG (32). To discover whether the upstream ORF plays any role in the translation of VA-Tat RNA, we eliminated it by mutating the start codon to GCG (mutant I) or extended it by 10 nt into the Tat ORF by mutating the upstream stop codon to CAG (mutant S) (Fig. 6A). The initiation codon mutant gave only twofold more CAT activity than wild-type pVA-Tat and the stop codon mutant gave twofold less activity than wild-type pVA-Tat in the HIV LTR transactivation assay (Fig. 6B), suggesting that the upstream ORF can be translated and that its utilization decreases Tat synthesis. Consistent with this conclusion, transactivation by the double mutant I+S was similar to that of the I mutant, indicating that the stop codon is influential only if the upstream ORF is utilized. These results indicate that scanning of the 5′ untranslated region may be involved in translation of the uncapped VA-Tat RNA.

DISCUSSION

It is an accepted fact that pol II produces mRNA precursors in eukaryotic cells. We have asked whether this marked functional specialization is obligatory or facultative; in other words, is the synthesis of mRNA necessarily and exclusively a pol II function? To address this question, we determined whether pol III can make functional mRNA by examining the transcriptional ability and translational potential of a chimera containing a protein-coding sequence under the control of a pol III

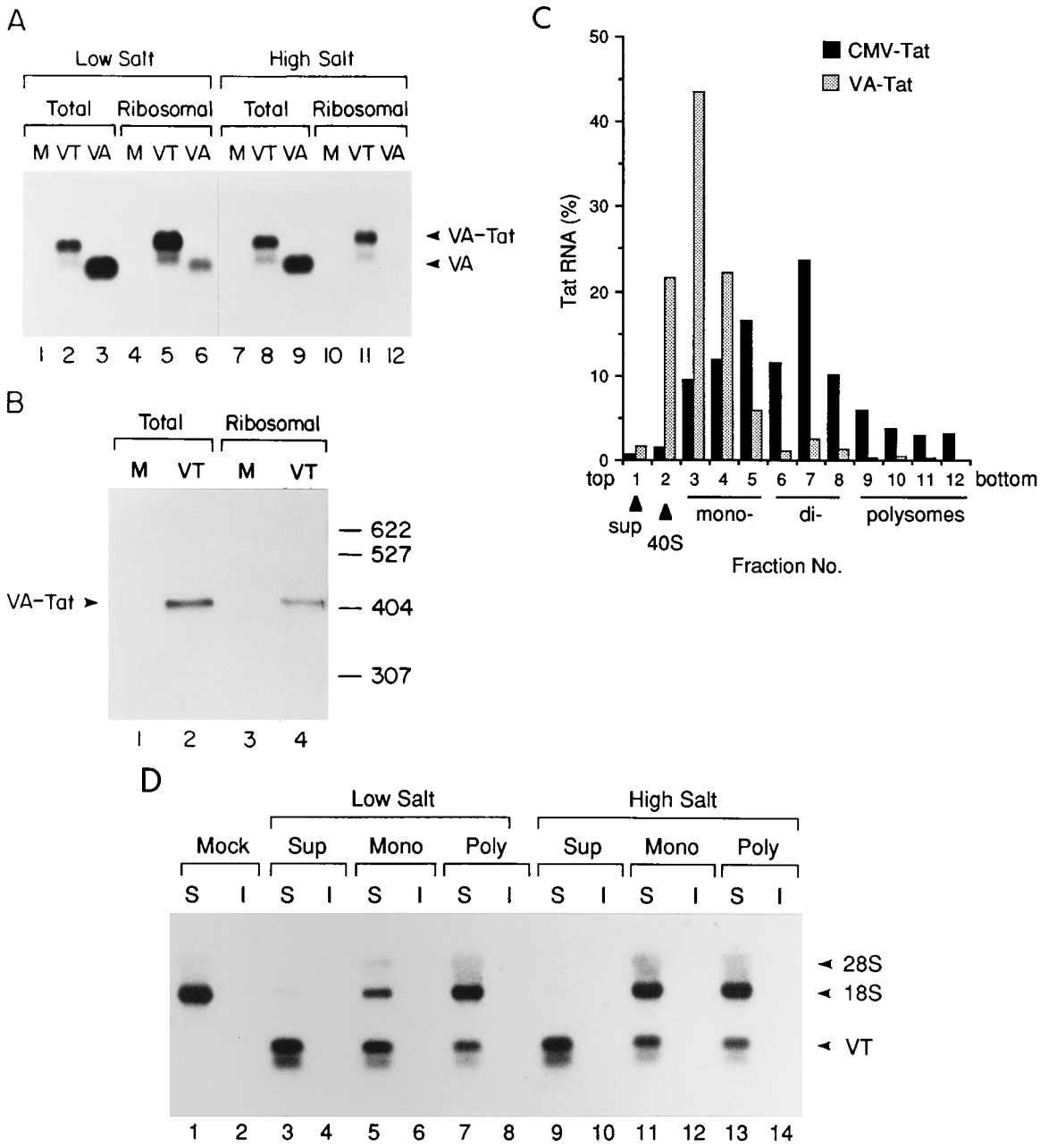


FIG. 6. Polysome association of VA-Tat RNA. (A) The lysate from cells transfected with pVA-Tat (VT) or pVA (VA) or from mock-transfected cells (M) was fractionated into ribosomal and cytosolic components under low- or high-salt conditions. RNA from the total lysate or the ribosomal fraction was subjected to Northern blot analysis with the 5′ VA probe. The positions of VA RNA and VA-Tat RNA are marked. (B) RNA from the total lysate or the ribosomal pellet was labeled in the capping reaction, hybrid selected for VA sequences, and analyzed in an 8% polyacrylamide–7 M urea gel. VT, VA-Tat RNA; M, mock-transfected cells. End-labeled DNA markers (in nucleotides) are given on the right. (C) Sucrose gradient analysis of polysomes from transfected cells. The lysate prepared from cells transfected with pVA-Tat or pCMV-Tat was fractionated in a sucrose gradient under high-salt conditions. RNA extracted from the fractions collected was subjected to RNase protection analysis with a probe complementary to the *tat* sequence. The protected product was quantified with a Fuji Phospho Imager. The positions of the supernatant (sup), 40S subunit, monosomes (mono-), disomes (di-), and larger polysomes are indicated. (D) Polysome-associated VA-Tat RNA is not capped. Lysates of cells transfected with pVA-Tat (VT) were resolved in sucrose gradients run under high-salt or low-salt conditions. The fractions containing the supernatant (Sup), monosomes (Mono), and polysomes (Poly) were pooled. RNA extracted from these fractions was subjected to immunoprecipitation with the cap antibodies (lanes I) and analyzed by Northern blot analysis with the 5′ VA probe. Lanes marked "S" contain the starting material.

promoter. Our results demonstrate that RNA transcribed by pol III can indeed serve as mRNA and give rise to a functional protein in the absence of a 5′ cap, a 3′ poly(A) tail, and intron removal, which have been proposed to be indispensable features of eukaryotic mRNA. A combination of biochemical and mutational evidence established that the transcript is a genuine product of pol III transcription rather than a product of transcription from a cryptic pol II promoter. Mutants targeting the VA RNA gene promoter elements or inserting termination signals specific for pol III had the expected effects on Tat synthesis, while biochemical analysis demonstrated that the VA-Tat RNA was nonpolyadenylated and uncapped, as ex-

pected of a pol III transcript. Moreover, polysome-associated VA-Tat RNA was uncapped, confirming that the pol III transcript was indeed translated. The results obtained in this model system have implications for mRNA synthesis and utilization, as well as for RNA polymerase selectivity.

mRNA production. Despite the structural and functional distinctions between the three types of cellular RNA polymerase, some common features have come to light in recent years. For example, the TATA-element-binding protein is essential for the function of all three polymerases (for a review, see reference 24), and one class of pol III promoters is so similar in primary structure to a pol II promoter that its specificity can be switched by the elimination of a functional TATA element (39). At the level of processing, although some studies have shown that splicing of RNA is coupled to pol II transcription (64), other studies have shown that these two processes can be dissociated (20, 34). Moreover, polyadenylation, which was thought to be undertaken only by pol II transcripts, has been reported for naturally occurring pol III transcripts in murine tumor cells (35, 36, 63) as well as for a chimeric RNA transcribed by pol III (38). A low-abundancy β-globin transcript that initiates upstream of the normal β-globin mRNA initiation site and is transcribed by pol III has been reported to be present in bone marrow cells and in peripheral blood reticulocytes (8). These pol III transcripts are polyadenylated and spliced correctly, like the normal β-globin mRNA. Taken together, these observations provide some precedents, albeit fragmentary, for the synthesis of mRNA by pol III reported here. Furthermore, Alu elements, which are transcribed by pol III, are found within protein-coding sequences (7, 42, 61, 72). Nevertheless, as far as we are aware, no cellular mRNA has been shown to be produced by a polymerase other than pol II.

On the other hand, many viruses make mRNA by using viral RNA polymerases, circumventing the highly specialized pathways for mRNA processing, transport, and utilization employed by cellular genes. Some viruses, such as vaccinia virus, reovirus, and poliovirus, have resorted to transcription in the cytoplasm of the host cell to bypass the processing and transportation complication. The transcripts of these viruses are capped by virus-specific enzymes and translated like cellular pol II transcripts. Other viral genomes, like that of influenza virus, are transcribed in the nucleus and the transcripts are correctly transported and translated, implying that the specificity of the pathways is not rigorously maintained or that viral mRNAs gain access to the normal pathway at intermediate points. Evidently, pol III transcripts can enter these pathways or utilize alternative routes leading from the nucleus to the polysome. From the results shown in Fig. 2, however, it could appear that expression of Tat from pVA-Tat saturates at a relatively low level, despite the accumulation of the pol III transcript to high levels. Thus, there seems to be a bottleneck(s) at one or more steps in the pathway.

Translational initiation. All known cellular mRNAs are capped, and most of them are spliced and polyadenylated. According to the scanning hypothesis, the cap plays an important role in translational initiation: the 40S ribosomal subunit is thought to bind at the 5′ end of the RNA, an event which is facilitated by the cap and a cap-binding initiation factor (eIF-4E), and then to migrate along the RNA until it encounters the first AUG codon, at which it initiates translation (for a review, see reference 32). Three kinds of exceptions to this model are recognized: the first AUG can be bypassed if it is in a suboptimal sequence context or duplex structure, its utilization can be influenced by initiation factors and conditioned by prior short ORFs (18; for a review, see reference 33), and it can be disregarded if the RNA contains an IRES (for a review, see reference 66). IRES elements are found in uncapped picornavirus RNAs and even in the naturally capped mRNAs of hepatitis C virus and some cellular mRNAs which have a longer-than-average 5′ untranslated region. The IRES confers cap-independent translation upon the mRNA, playing a role analogous to that of the cap structure in binding the 40S ribosomal subunit to the mRNA for translation but permitting initiation at a downstream AUG codon. A more recent scanning model (66) proposes that initiation factor complexes, rather than ribosomal subunits, scan the 5′ region and advances the idea that scanning may not be 5′ end dependent. Scanning by the initiation factors is proposed to cause unwinding of the RNA, thereby creating a site for ribosome binding and leading to initiation. Whatever the mechanism, our data show that the presence of a cap is not essential.

Under some circumstances at least, the poly(A) tail found at the 3′ end of most eukaryotic mRNAs is required for mRNA stability, its transport into the cytoplasm, and its efficient translation (for a review, see reference 29). In particular, a poly(A) tail has been implicated in the initiation of translation (48). The poly(A)-binding protein, which is present in almost all eukaryotic cells and which forms a complex with the poly(A) tail, also enhances initiation (59), supporting the view that the poly(A) tail plays a role in translation. On the other hand, there are observations that argue against the universality of the stimulatory role of poly(A) in translation. For example, a deficiency of poly(A)-binding protein or lack of poly(A) tails did not significantly affect the translational efficiency of yeast mRNAs (53); deadenylated zein mRNA is translated 50% as efficiently as poly(A)$^+$ mRNA in microinjected *Xenopus* oocytes (16), and deadenylation of histone mRNA actually facilitates translation of histone messages in *Xenopus* oocytes (3). Our results show that the translation of the pol III transcript can occur in the absence of a poly(A) tail, but further studies are needed to determine if polyadenylation of the pol III transcript can improve its translational efficiency.

VA-Tat RNA contains a short 5′ ORF of 45 nt upstream of the Tat ORF, with 37 nt intervening between them. A mutation in the upstream AUG, which in effect abolished the short ORF, resulted in a modest increase in Tat transactivation, and mutation of the stop codon of the upstream ORF gave only a twofold decrease in transactivation (Fig. 7). Although these effects are in principle compatible with scanning, their magnitude was small. At least three explanations can be entertained. First, it is possible that the first AUG is "leaky" (allowing bypass), even though it is in a good context for initiation (AUCAUGG), perhaps because the fidelity of the scanning mechanism is lower if a cap is absent. Leaky initiation codons that are present in a favorable context, although uncommon, have been reported earlier (12, 49). Second, under certain circumstances ribosomes can reinitiate at a downstream AUG after terminating at an upstream stop codon (27, 31). However, the intercistronic sequence of VA-Tat RNA may be too short for efficient reinitiation (31). Mutation at site S extends the upstream ORF so that it overlaps the Tat ORF by 10 nt. In light of the finding by Peabody et al. (50) that ribosomes can scan backward for up to 50 nt and reinitiate at the AUG of the downstream ORF, it is conceivable that reinitiation at the downstream AUG occurs by back scanning after translation of the upstream ORF terminates. Increasing the overlap of the two ORFs reduced translation of the downstream *tat* ORF, indicating back scanning as a strong possibility in mutant S (unpublished data). Third, perhaps ribosomes bind to uncapped mRNA in a random fashion and migrate to the nearest AUG, where they initiate translation. Those ribosomes that bind between the two AUGs would not be affected by muta-

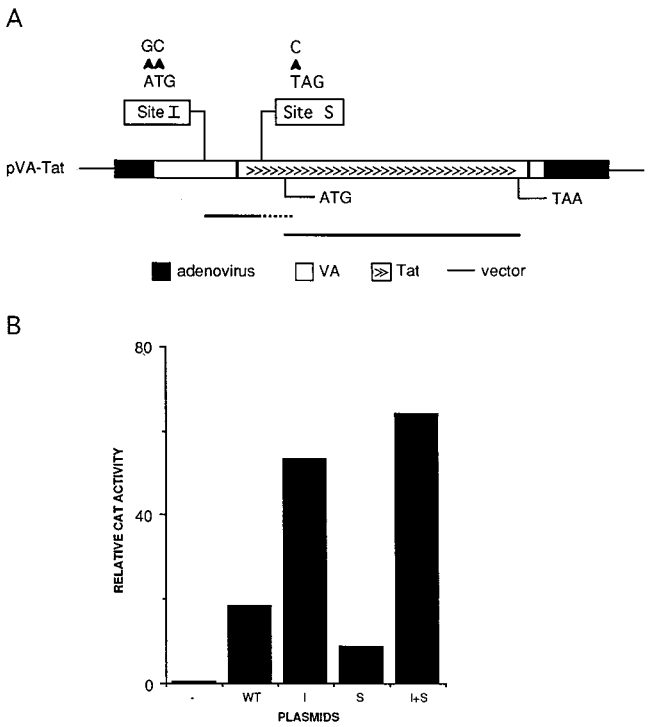


FIG. 7. Effect of the upstream ORF on expression of the downstream *tat* ORF. (A) Sites of initiator codon (I) and stop codon (S) mutations. ORFs are indicated at the bottom by solid lines, and the dotted line indicates the extension caused by the S mutation. (B) pHIV-CAT transactivation by wild-type (WT) pVA-Tat and/or mutant pVA-Tat (I, S, or I+S) in transfected cells. The relative CAT enzyme activity is shown as CAT units per microliter of the lysate. The bar labeled with the minus sign indicates basal activity from pHIV-CAT alone.

tions that eliminate the upstream ORF (mutants I and I+S) or by those that extend the ORF into the Tat ORF (mutant S), thereby accounting for the relatively small effect of these mutations. Although the existence of an IRES in the 5′ untranslated region of the RNA has not yet been ruled out, the small size and lack of secondary structure in the 5′ untranslated region make it unlikely.

Polymerase selectivity. The demonstration that mRNA can be produced by pol III raises two related questions: are any cellular mRNAs made by pol I or pol III, and how and why do cells preserve pol II as the predominant, if not exclusive, source of mRNA? The maintenance of specificity may confer advantages, such as correct processing and transportation, that allow an efficient utilization of the transcript and may even provide specific signals for regulation of gene expression. Because an artificial pol III transcript can also be translated, cells may also impose functional limitations that maintain the distinct specialization of the polymerases. One of the obvious limitations in the case of pol III is the requirement that the RNA be free of runs of T residues, but the degeneracy of the genetic code is such that this obstacle should not be insuperable. Moreover, our data indicate that the termination signal may be more complex than previously suspected, since, for reasons not understood, pol III ignored a stop signal (mutant T1) even though it was surrounded by GC-rich sequences. Some yeast viral mRNAs are uncapped (6), and their translation is suppressed by the yeast chromosomal gene *SKI2*, which is proposed to be a part of a cellular defense mechanism (71). *SKI2* blocks the translation of uncapped messages only, and disruption of the *SKI2* gene led to increased translation of a pol I-directed mRNA which supposedly is uncapped. Such cellular defense mechanisms against viral infection may also impose limitations that maintain the specificity of the polymerases.

It is also possible that the promoter sequence dictates polymerase selection, which in turn leads the transcript through a specific pathway for processing and transport by compartmentalization and/or choice of proteins that are associated with the transcript. If preferences for pol II transcripts are exerted at different levels (processing, nuclear transport and export, ribosome binding, etc.) the combined effect may be sufficient to effectively preclude mRNA synthesis by other polymerases. On the other hand, it is possible that circumstances exist—for example, during selected stages of development or phases of the cell cycle or in unusual physiological states or virus infections—under which the pol II-coupled pathway of gene expression is inefficient or inactivated. Under such conditions, the ability to generate mRNA by an alternative route might be advantageous. This idea could explain the otherwise-surprising observation that transcription from the adenovirus E2E promoter (28, 54), c-*myc* P1 and P2 promoters (4, 9), and human T-cell lymphotrophic virus type 1 promoter (52) can be initiated by both pol II and pol III from a common start site. Furthermore, there might be conditions under which uncapped mRNA is preferentially used for protein synthesis, for instance, when cap-binding protein is limiting. Under such circumstances, those RNAs that can initiate without a cap could be selectively translated.

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